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FIG. 1A

2H7scFv-Ig cDNA and predicted amino acid sequence:

HindIII NcoI 2H7 V_L Leader Peptide→

```
                                M  D  F  Q  V  Q  I  F  S  F  L  L  I  S  A  S
1   AAGCTTGCCG CC   ATGGATTT TCAAGTGCAG ATTTTCAGCT TCCTGCTAAT CAGTGCTTCA

                                                2H7 VL→
    V  I  I  A  R  G  Q  I  V  L  S  Q  S  P  A  I  L  S  A  S
61  GTCATAATTG CCAGAGGACA AATTGTTCTC TCCCAGTCTC CAGCAATCCT GTCTGCATCT

    P  G  E  K  V  T  M  T  C  R  A  S  S  S  V  S  Y  M  H  W
121 CCAGGGGAGA AGGTCACAAT GACTTGCAGG GCCAGCTCAA GTGTAAGTTA CATGCACTGG

                    BamHI
                    ~~~~~~~
    Y  Q  Q  K  P  G  S  S  P  K  P  W  I  Y  A  P  S  N  L  A
181 TACCAGCAGA AGCCAGGATC CTCCCCCAAA CCCTGGATTT ATGCCCCATC CAACCTGGCT

    S  G  V  P  A  R  F  S  G  S  G  S  G  T  S  Y  S  L  T  I
241 TCTGGAGTCC CTGCTCGCTT CAGTGGCAGT GGGTCTGGGA CCTCTTACTC TCTCACAATC

    S  R  V  E  A  E  D  A  A  T  Y  Y  C  Q  Q  W  S  F  N  P
301 AGCAGAGTGG AGGCTGAAGA TGCTGCCACT TATTACTGCC AGCAGTGGAG TTTTAACCCA

(Gly4Ser)3 Linker
    P  T  F  G  A  G  T  K  L  E  L  K  G  G  G  G  S  G  G  G
361 CCCACGTTCG GTGCTGGGAC CAAGCTGGAG CTGAAAGGTG GCGGTGGCTC GGGCGGTGGT

                                                2H7 VH →
    G  S  G  G  G  G  S  S  Q  A  Y  L  Q  Q  S  G  A  E  L  V
421 GGATCTGGAG GAGGTGGGAG CTCTCAGGCT TATCTACAGC AGTCTGGGGC TGAGCTGGTG

    R  P  G  A  S  V  K  M  S  C  K  A  S  G  Y  T  F  T  S  Y
481 AGGCCTGGGG CCTCAGTGAA GATGTCCTGC AAGGCTTCTG GCTACACATT TACCAGTTAC

    N  M  H  W  V  K  Q  T  P  R  Q  G  L  E  W  I  G  A  I  Y
541 AATATGCACT GGGTAAAGCA GACACCTAGA CAGGGCCTGG AATGGATTGG AGCTATTTAT

    P  G  N  G  D  T  S  Y  N  Q  K  F  K  G  K  A  T  L  T  V
601 CCAGGAAATG GTGATACTTC CTACAATCAG AAGTTCAAGG GCAAGGCCAC ACTGACTGTA

    D  K  S  S  S  T  A  Y  M  Q  L  S  S  L  T  S  E  D  S  A
661 GACAAATCCT CCAGCACAGC CTACATGCAG CTCAGCAGCC TGACATCTGA AGACTCTGCG

    V  Y  F  C  A  R  V  V  Y  Y  S  N  S  Y  W  Y  F  D  V  W
721 GTCTATTTCT GTGCAAGAGT GGTGTACTAT AGTAACTCTT ACTGGTACTT CGATGTCTGG
```

FIG.1B

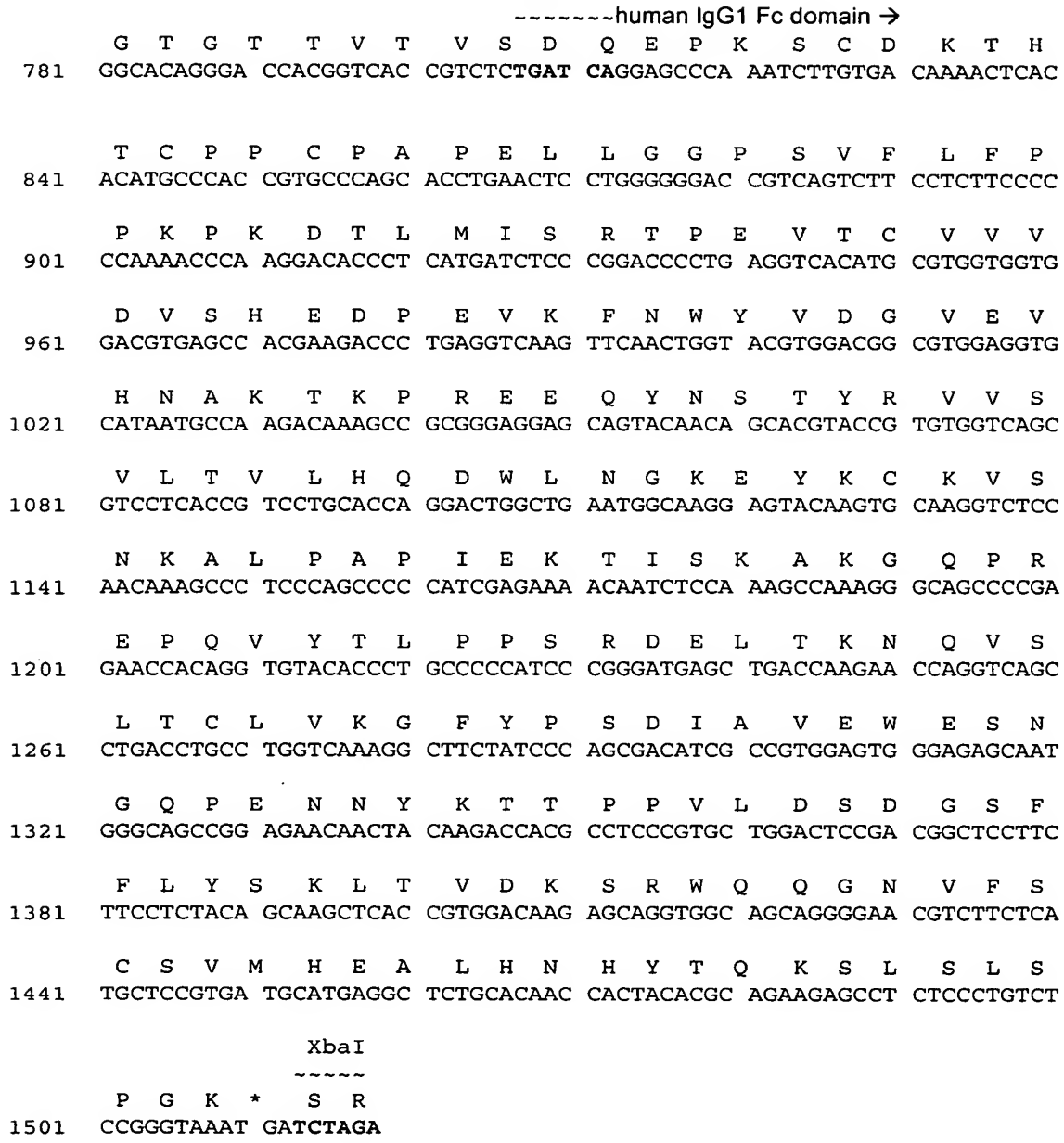
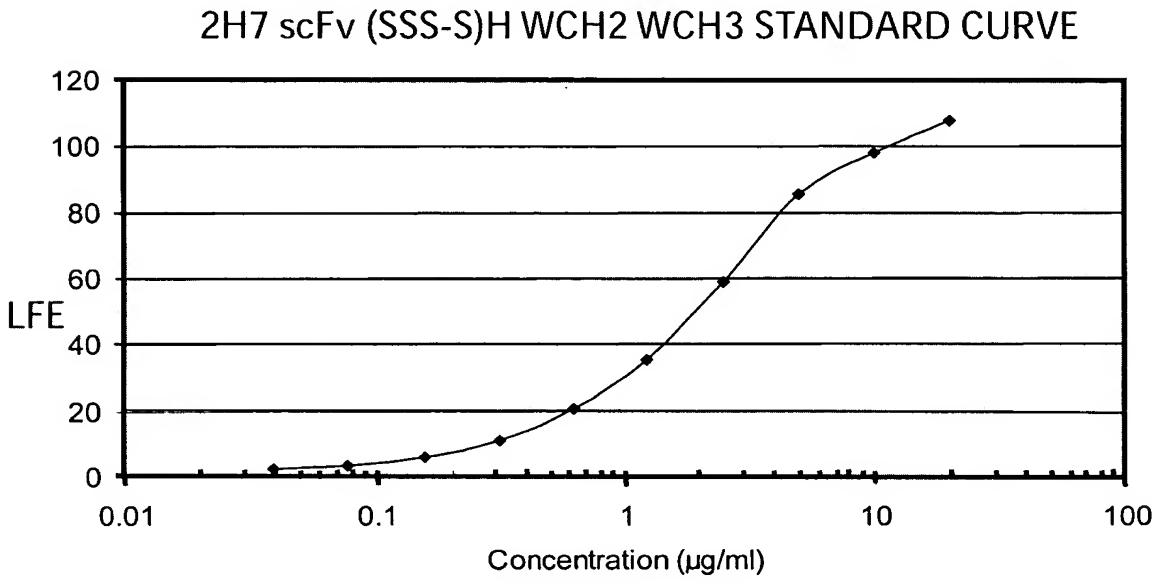

BclI
~~~~~~~human IgG1 Fc domain →
G T G T T V T V S D Q E P K S C D K T H
781 GGCACAGGGA CCACGGTCAC CGTCTCTGAT CAGGAGCCCA AATCTTGTGA CAAAACTCAC
T C P P C P A P E L L G G P S V F L F P
841 ACATGCCCAC CGTGCCCAGC ACCTGAACTC CTGGGGGGAC CGTCAGTCTT CCTCTTCCCC
P K P K D T L M I S R T P E V T C V V V
901 CCAAAACCCA AGGACACCCT CATGATCTCC CGGACCCCTG AGGTCACATG CGTGGTGGTG
D V S H E D P E V K F N W Y V D G V E V
961 GACGTGAGCC ACGAAGACCC TGAGGTCAAG TTCAACTGGT ACGTGGACGG CGTGGAGGTG
H N A K T K P R E E Q Y N S T Y R V V S
1021 CATAATGCCA AGACAAAGCC GCGGGAGGAG CAGTACAACA GCACGTACCG TGTGGTCAGC
V L T V L H Q D W L N G K E Y K C K V S
1081 GTCCTCACCG TCCTGCACCA GGACTGGCTG AATGGCAAGG AGTACAAGTG CAAGGTCTCC
N K A L P A P I E K T I S K A K G Q P R
1141 AACAAAGCCC TCCCAGCCCC CATCGAGAAA ACAATCTCCA AAGCCAAAGG GCAGCCCCGA
E P Q V Y T L P P S R D E L T K N Q V S
1201 GAACCACAGG TGTACACCCT GCCCCCATCC CGGGATGAGC TGACCAAGAA CCAGGTCAGC
L T C L V K G F Y P S D I A V E W E S N
1261 CTGACCTGCC TGGTCAAAGG CTTCTATCCC AGCGACATCG CCGTGGAGTG GGAGAGCAAT
G Q P E N N Y K T T P P V L D S D G S F
1321 GGGCAGCCGG AGAACAACTA CAAGACCACG CCTCCCGTGC TGGACTCCGA CGGCTCCTTC
F L Y S K L T V D K S R W Q Q G N V F S
1381 TTCCTCTACA GCAAGCTCAC CGTGGACAAG AGCAGGTGGC AGCAGGGGAA CGTCTTCTCA
C S V M H E A L H N H Y T Q K S L S L S
1441 TGCTCCGTGA TGCATGAGGC TCTGCACAAC CACTACACGC AGAAGAGCCT CTCCCTGTCT
XbaI
~~~~~
P G K * S R
1501 CCGGGTAAAT GATCTAGA

FIG.2

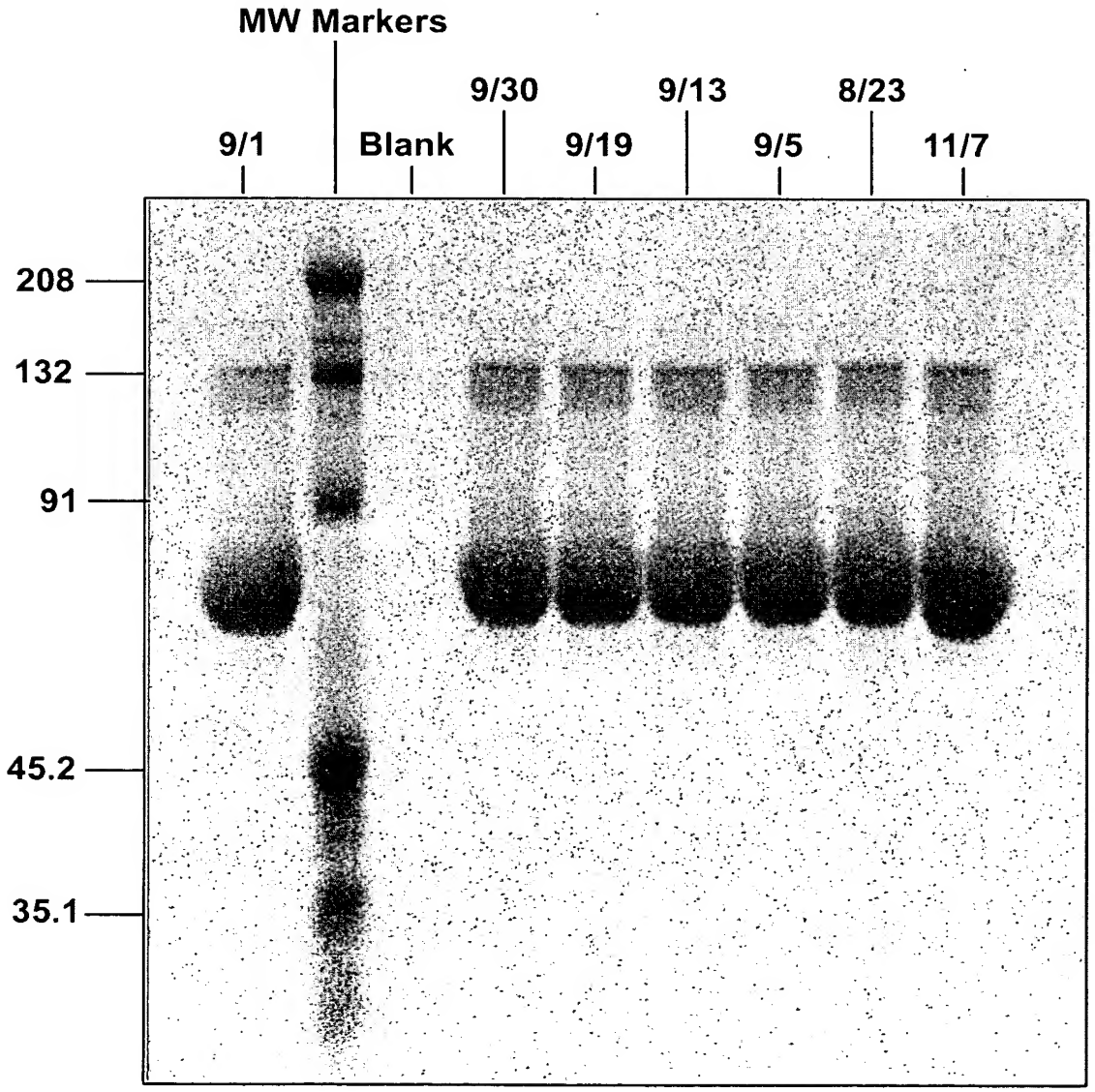
Production Levels of 2H7 scFv (SSS-S)H WCH2 WCH3
by Stable CHO Lines




| Clone | LFE @ 1:50 Estimated Concentration (mg/ml) |
|---|---|
| D2 | 26.156 |
| IIIC6 | 25.755 |
| IVA3 | 28.661 |
| Spent bulk | 29.664 |

FIG.3

SDS-Page Analysis of
2H7 scFv (SSS-S)H WCH2 WCH3 Protein



# FIG.4A

**Complement Mediated B Cell Killing After Binding of CD20-targeted 2H7 scFv (SSS-S)H WCH2 WCH3**

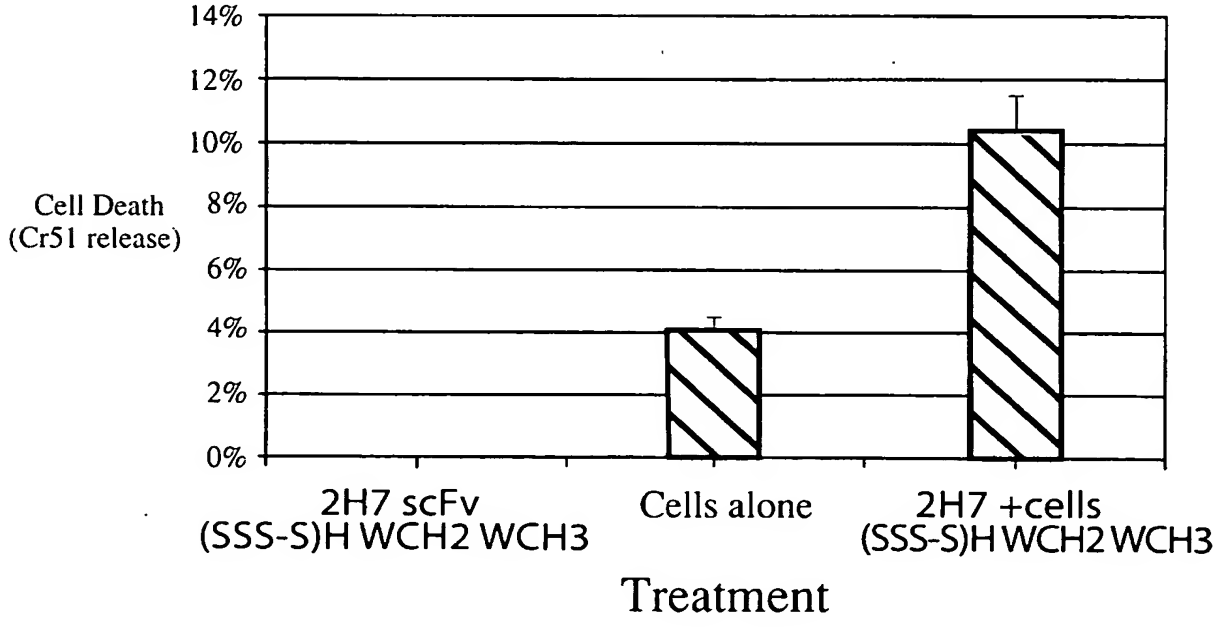
| 2H7scFv-Ig Concentration | RAMOS # LIVE CELLS / TOTAL CELLS | BJAB # LIVE CELLS / TOTAL CELLS |
|---|---|---|
| 20 μg/ml + complement | 0.16 | 0.07 |
| 5 μg/ml + complement | 0.2 | N.D. |
| 1.25 μg/ml + complement | 0.32 | 0.1 |
| Complement alone | 0.98 | 0.94 |

*Viability was determined by trypan blue exclusion and is tabulated as the fraction of viable cells out of the total number of cells counted.

**N.D. (not determined).

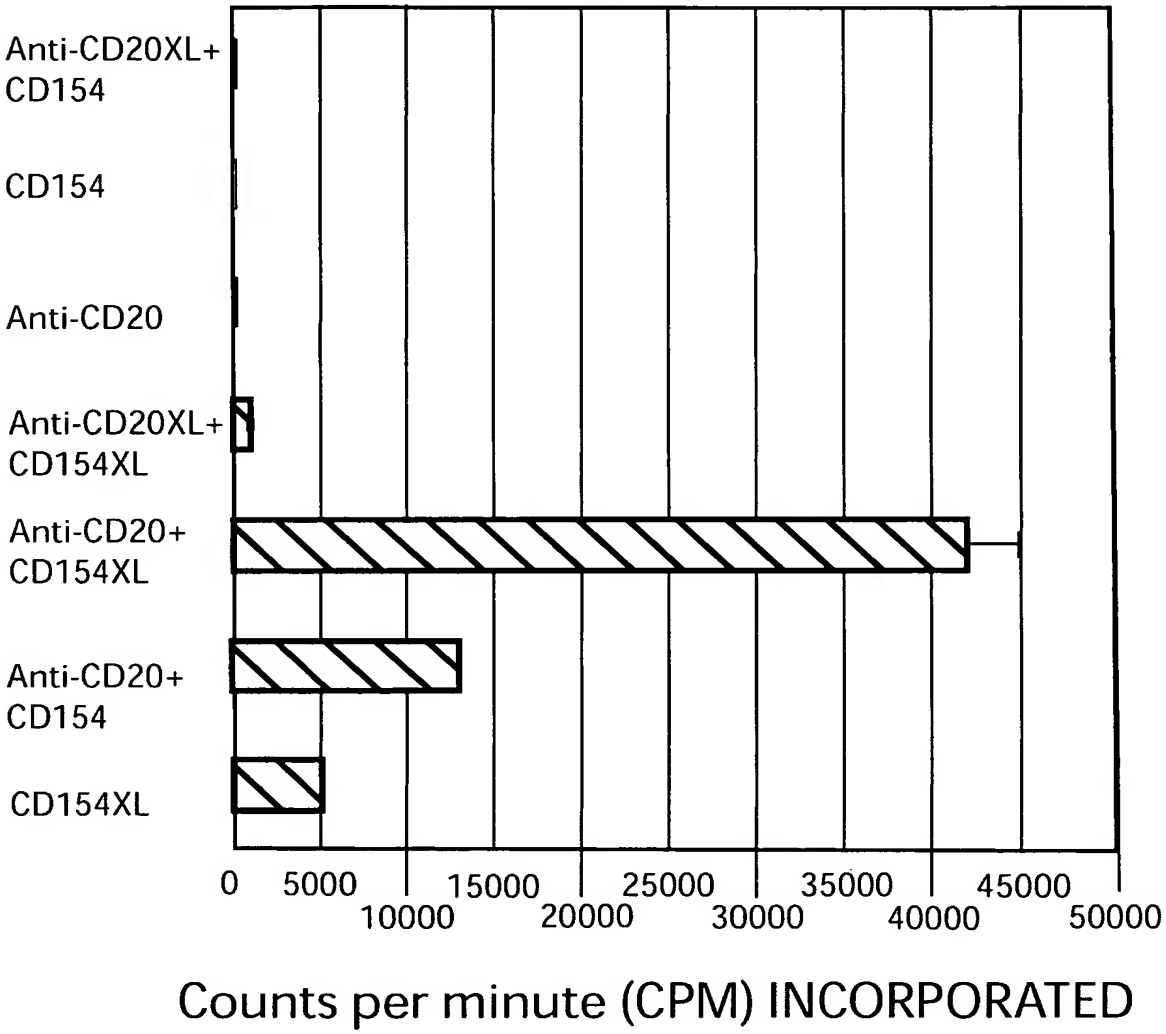
# FIG.4B

Antibody-dependent cellular cytotoxicity (ADCC) mediated by 2H7 scFv (SSS-S)H WCH2 WCH3



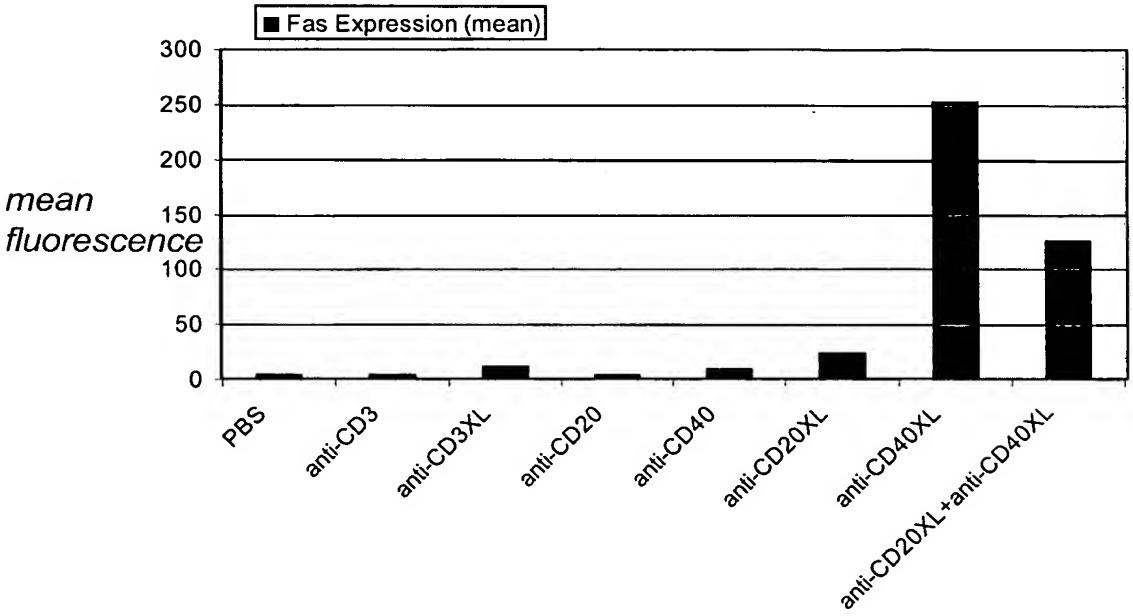
# FIG.5

Effects of Crosslinking of CD20 and CD40 Cell Surface Receptors on B Cell Proliferation:



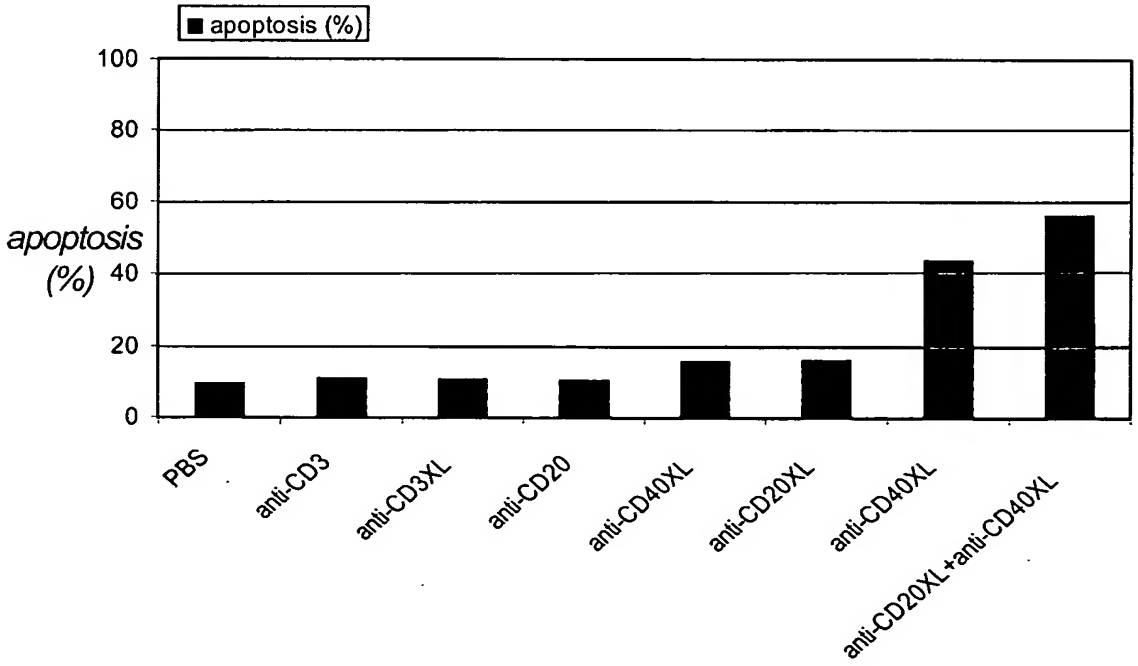
# FIG.6A

*Effect of Simultaneous ligation of CD20 and CD40 on CD95 and apoptosis.*




# FIG.6B

*Effect of Simultaneous ligation of CD20 and CD40 on CD95 and apoptosis.*



# FIG. 7A

2H7-CD154 L2 cDNA and predicted amino acid sequence:

HindIII NcoI 2H7 $V_L$ Leader Peptide →

```
                                M  D  F  Q  V  Q  I  F  S  F  L  L  I  S  A  S
1   AAGCTTGCCG CC   ATGGATTT TCAAGTGCAG ATTTTCAGCT TCCTGCTAAT CAGTGCTTCA

                                          2H7 VL →
     V  I  I  A  R  G  Q  I  V  L  S  Q  S  P  A  I  L  S  A  S
61  GTCATAATTG CCAGAGGACA AATTGTTCTC TCCCAGTCTC CAGCAATCCT GTCTGCATCT

     P  G  E  K  V  T  M  T  C  R  A  S  S  S  V  S  Y  M  H  W
121 CCAGGGGAGA AGGTCACAAT GACTTGCAGG GCCAGCTCAA GTGTAAGTTA CATGCACTGG

                    BamHI
                    -------
     Y  Q  Q  K  P  G  S  S  P  K  P  W  I  Y  A  P  S  N  L  A
181 TACCAGCAGA AGCCAGGATC CTCCCCCAAA CCCTGGATTT ATGCCCCATC CAACCTGGCT

     S  G  V  P  A  R  F  S  G  S  G  S  G  T  S  Y  S  L  T  I
241 TCTGGAGTCC CTGCTCGCTT CAGTGGCAGT GGGTCTGGGA CCTCTTACTC TCTCACAATC

     S  R  V  E  A  E  D  A  A  T  Y  Y  C  Q  Q  W  S  F  N  P
301 AGCAGAGTGG AGGCTGAAGA TGCTGCCACT TATTACTGCC AGCAGTGGAG TTTTAACCCA

(Gly4Ser)3 Linker →
     P  T  F  G  A  G  T  K  L  E  L  K  G  G  G  G  S  G  G  G
361 CCCACGTTCG GTGCTGGGAC CAAGCTGGAG CTGAAAGGTG GCGGTGGCTC GGGCGGTGGT

                                                      2H7 VH →
     G  S  G  G  G  G  S  S  Q  A  Y  L  Q  Q  S  G  A  E  L  V
421 GGATCTGGAG GAGGTGGGAG CTCTCAGGCT TATCTACAGC AGTCTGGGGC TGAGCTGGTG

     R  P  G  A  S  V  K  M  S  C  K  A  S  G  Y  T  F  T  S  Y
481 AGGCCTGGGG CCTCAGTGAA GATGTCCTGC AAGGCTTCTG GCTACACATT TACCAGTTAC

     N  M  H  W  V  K  Q  T  P  R  Q  G  L  E  W  I  G  A  I  Y
541 AATATGCACT GGGTAAAGCA GACACCTAGA CAGGGCCTGG AATGGATTGG AGCTATTTAT

     P  G  N  G  D  T  S  Y  N  Q  K  F  K  G  K  A  T  L  T  V
601 CCAGGAAATG GTGATACTTC CTACAATCAG AAGTTCAAGG GCAAGGCCAC ACTGACTGTA

     D  K  S  S  S  T  A  Y  M  Q  L  S  S  L  T  S  E  D  S  A
661 GACAAATCCT CCAGCACAGC CTACATGCAG CTCAGCAGCC TGACATCTGA AGACTCTGCG

     V  Y  F  C  A  R  V  V  Y  Y  S  N  S  Y  W  Y  F  D  V  W
721 GTCTATTTCT GTGCAAGAGT GGTGTACTAT AGTAACTCTT ACTGGTACTT CGATGTCTGG
```

# FIG.7B

human CD154/amino acid 48→

site Bcl/Bam hybrid

```
      G  T  G  T  T  V  T  V  S  D  P  R  R  L  D  K  I  E  D  E
781   GGCACAGGGA CCACGGTCAC CGTCTC***TGAT CC***AAGAAGGT TGGACAAGAT AGAAGATGAA

      R  N  L  H  E  D  F  V  F  M  K  T  I  Q  R  C  N  T  G  E
841   AGGAATCTTC ATGAAGATTT TGTATTCATG AAAACGATAC AGAGATGCAA CACAGGAGAA

      R  S  L  S  L  L  N  C  E  E  I  K  S  Q  F  E  G  F  V  K
901   AGATCCTTAT CCTTACTGAA CTGTGAGGAG ATTAAAAGCC AGTTTGAAGG CTTTGTGAAG

                                                                  BclI
      D  I  M  L  N  K  E  E  T  K  K  E  N  S  F  E  M  Q  K  G
961   GATATAATGT TAAACAAAGA GGAGACGAAG AAAGAAAACA GCTTTGAAAT GCAAAAAGGT

      BclI
      ~~~~~
      D Q N P Q I A A H V I S E A S S K T T S
1021 GATCAGAATC CTCAAATTGC GGCACATGTC ATAAGTGAGG CCAGCAGTAA AACAACATCT

      V L Q W A E K G Y Y T M S N N L V T L E
1081 GTGTTACAGT GGGCTGAAAA AGGATACTAC ACCATGAGCA ACAACTTGGT AACCCTGGAA

      N G K Q L T V K R Q G L Y Y I Y A Q V T
1141 AATGGGAAAC AGCTGACCGT TAAAAGACAA GGACTCTATT ATATCTATGC CCAAGTCACC

                  HindIII
                  ~~~~~~~
      F C S N R E A S S Q A P F I A S L C L K
1201 TTCTGTTCCA ATCGGGAAGC TTCGAGTCAA GCTCCATTTA TAGCCAGCCT CTGCCTAAAG

      S P G R F E R I L L R A A N T H S S A K
1261 TCCCCCGGTA GATTCGAGAG AATCTTACTC AGAGCTGCAA ATACCCACAG TTCCGCCAAA

      P C G Q Q S I H L G G V F E L Q P G A S
1321 CCTTGCGGGC AACAATCCAT TCACTTGGGA GGAGTATTTG AATTGCAACC AGGTGCTTCG

                                          NcoI
                                          ~~~~~~~
      V F V N V T D P S Q V S H G T G F T S F
1381 GTGTTTGTCA ATGTGACTGA TCCAAGCCAA GTGAGCCATG GCACTGGCTT CACGTCCTTT

                  XhoI   XbaI
                  ~~~~~~   ~~~~
      G L L K L E * * S R
1441 GGCTTACTCA AACTCGAGTG ATAA**TCTAGA**
```

FIG.7C

2H7scFv-CD154 S4 cDNA and predicted amino acid sequence:

HindIII NcoI
~~~~~~ ~~~~~~2H7 V_L Leader Peptide→

```
                    M  D  F  Q  V  Q  I  F  S  F  L  L  I  S  A  S
1   AAGCTTGCCG CC   ATGGATTT TCAAGTGCAG ATTTTCAGCT TCCTGCTAAT CAGTGCTTCA

                                        2H7 VL →
    V  I  I  A  R  G  Q  I  V  L  S  Q  S  P  A  I  L  S  A  S
61  GTCATAATTG CCAGAGGACA AATTGTTCTC TCCCAGTCTC CAGCAATCCT GTCTGCATCT

    P  G  E  K  V  T  M  T  C  R  A  S  S  S  V  S  Y  M  H  W
121 CCAGGGGAGA AGGTCACAAT GACTTGCAGG GCCAGCTCAA GTGTAAGTTA CATGCACTGG

                    BamHI
                    ~~~~~~~
    Y  Q  Q  K  P  G  S  S  P  K  P  W  I  Y  A  P  S  N  L  A
181 TACCAGCAGA AGCCAGGATC CTCCCCCAAA CCCTGGATTT ATGCCCCATC CAACCTGGCT

    S  G  V  P  A  R  F  S  G  S  G  S  G  T  S  Y  S  L  T  I
241 TCTGGAGTCC CTGCTCGCTT CAGTGGCAGT GGGTCTGGGA CCTCTTACTC TCTCACAATC

    S  R  V  E  A  E  D  A  A  T  Y  Y  C  Q  Q  W  S  F  N  P
301 AGCAGAGTGG AGGCTGAAGA TGCTGCCACT TATTACTGCC AGCAGTGGAG TTTTAACCCA

(Gly4Ser)3 Linker →
    P  T  F  G  A  G  T  K  L  E  L  K  G  G  G  G  S  G  G  G
361 CCCACGTTCG GTGCTGGGAC CAAGCTGGAG CTGAAAGGTG GCGGTGGCTC GGGCGGTGGT

                                                    2H7 VH →
    G  S  G  G  G  G  S  S  Q  A  Y  L  Q  Q  S  G  A  E  L  V
421 GGATCTGGAG GAGGTGGGAG CTCTCAGGCT TATCTACAGC AGTCTGGGGC TGAGCTGGTG

    R  P  G  A  S  V  K  M  S  C  K  A  S  G  Y  T  F  T  S  Y
481 AGGCCTGGGG CCTCAGTGAA GATGTCCTGC AAGGCTTCTG GCTACACATT TACCAGTTAC

    N  M  H  W  V  K  Q  T  P  R  Q  G  L  E  W  I  G  A  I  Y
541 AATATGCACT GGGTAAAGCA GACACCTAGA CAGGGCCTGG AATGGATTGG AGCTATTTAT

    P  G  N  G  D  T  S  Y  N  Q  K  F  K  G  K  A  T  L  T  V
601 CCAGGAAATG GTGATACTTC CTACAATCAG AAGTTCAAGG GCAAGGCCAC ACTGACTGTA

    D  K  S  S  S  T  A  Y  M  Q  L  S  S  L  T  S  E  D  S  A
661 GACAAATCCT CCAGCACAGC CTACATGCAG CTCAGCAGCC TGACATCTGA AGACTCTGCG

    V  Y  F  C  A  R  V  V  Y  Y  S  N  S  Y  W  Y  F  D  V  W
721 GTCTATTTCT GTGCAAGAGT GGTGTACTAT AGTAACTCTT ACTGGTACTT CGATGTCTGG
```
```

# FIG. 7D

human CD154/amino acid 108 →

```
      BclI   Bcl/Bam hybrid site
       G T G T   T V T   V S D   P E N S   F E M   Q K G
781   GGCACAGGGA CCACGGTCAC CGTCTC*TGAT* *CC*AGAAAACA GCTTTGAAAT GCAAAAAGGT

      BclI
      ~~~~~
       D Q N P   Q I A   A H V   I S E A   S S K   T T S
841   GATCAGAATC CTCAAATTGC GGCACATGTC ATAAGTGAGG CCAGCAGTAA AACAACATCT

       V L Q W   A E K   G Y Y   T M S N   N L V   T L E
901   GTGTTACAGT GGGCTGAAAA AGGATACTAC ACCATGAGCA ACAACTTGGT AACCCTGGAA

       N G K Q   L T V   K R Q   G L Y Y   I Y A   Q V T
961   AATGGGAAAC AGCTGACCGT TAAAAGACAA GGACTCTATT ATATCTATGC CCAAGTCACC

                    HindIII
                    ~~~~~~~
       F C S N   R E A   S S Q   A P F I   A S L   C L K
1021 TTCTGTTCCA ATCGGGAAGC TTCGAGTCAA GCTCCATTTA TAGCCAGCCT CTGCCTAAAG

       S P G R   F E R   I L L   R A A N   T H S   S A K
1081 TCCCCCGGTA GATTCGAGAG AATCTTACTC AGAGCTGCAA ATACCCACAG TTCCGCCAAA

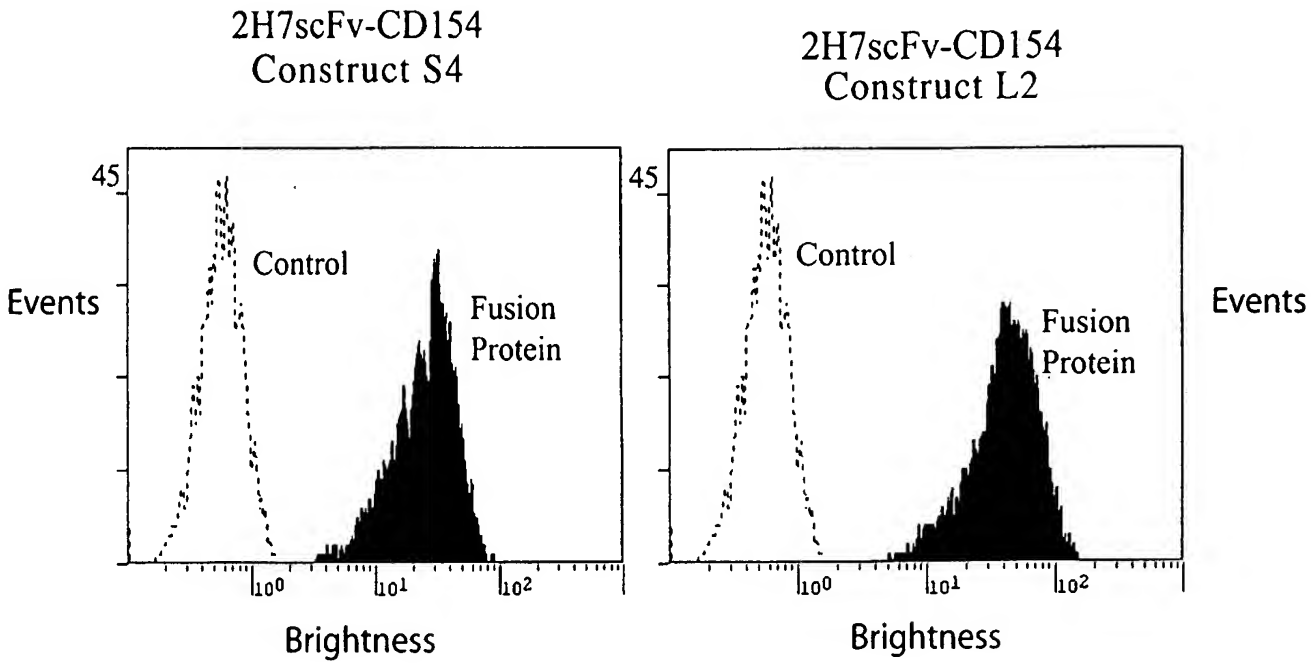
       P C G Q   Q S I   H L G   G V F E   L Q P   G A S
1141 CCTTGCGGGC AACAATCCAT TCACTTGGGA GGAGTATTTG AATTGCAACC AGGTGCTTCG

                                                NcoI
                                                ~~~~~~~
       V F V N   V T D   P S Q   V S H G   T G F   T S F
1201 GTGTTTGTCA ATGTGACTGA TCCAAGCCAA GTGAGCCATG GCACTGGCTT CACGTCCTTT

                    XhoI   XbaI
                    ~~~~~~~   ~~~~
       G L L K   L E *   * S R
1261 GGCTTACTCA AACTCGAGTG ATAATCTAGA
```

FIG.8

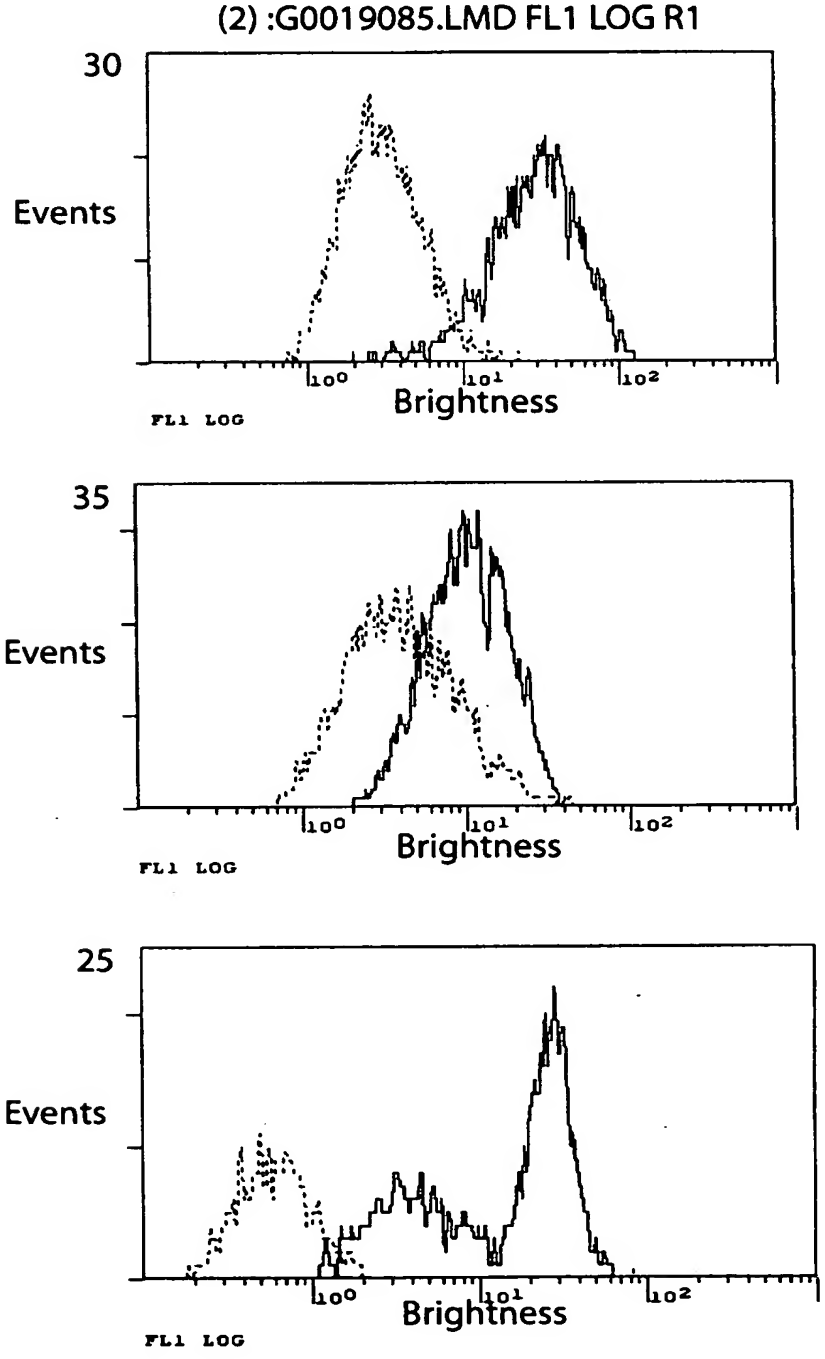
Simultaneous Binding of 2H7scFv-CD154
Fusion Proteins to CD20 and CD40




CD20 CHO cell targets + (control or fusion protein)
+ Biotin-CD40Ig + PE-SA

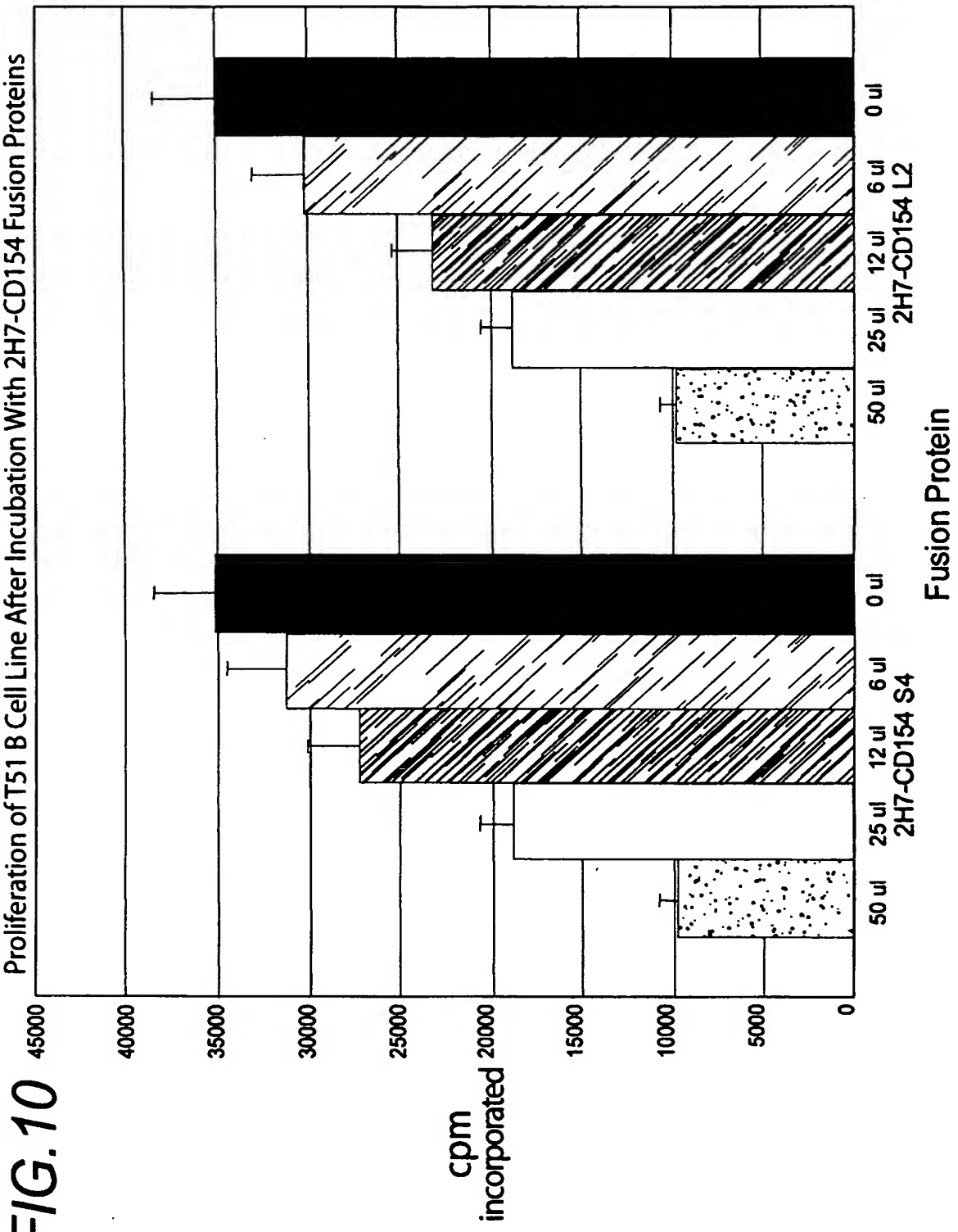
# FIG.9

Induction of Apoptosis Measured by Binding of Annexin V after incubation with 2H7scFv-CD154




.....:control supernatant ___2H7scFv-CD154 supernatant

FIG. 10


Proliferation of T51 B Cell Line After Incubation With 2H7-CD154 Fusion Proteins
cpm incorporated
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0 ul
6 ul
12 ul
25 ul
50 ul
2H7-CD154 S4
2H7-CD154 L2
Fusion Protein

# FIG. 11

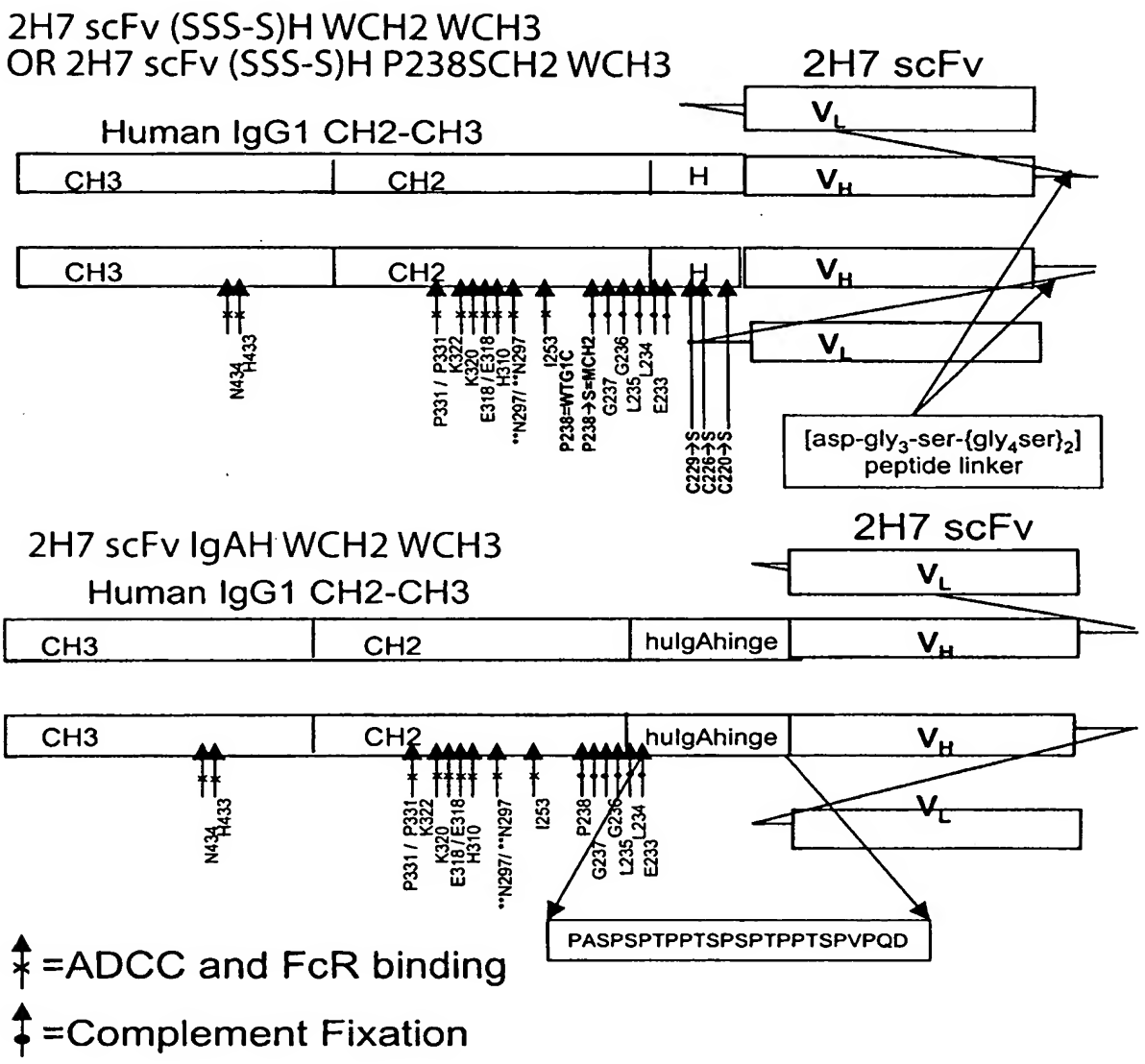

2H7 scFv (SSS-S)H WCH2 WCH3
OR 2H7 scFv (SSS-S)H P238SCH2 WCH3
2H7 scFv
Human IgG1 CH2-CH3
CH3
CH2
H
$V_L$
$V_H$
N434
H433
P331 / P331
K322
K320
E318 / E318
H310
**N297/ **N297
I253
P238=WTG1C
P238->S=MCH2
G237
G236
L235
L234
E233
C229->S
C226->S
C220->S
[asp-gly3-ser-{gly4ser}2]
peptide linker
2H7 scFv IgAH WCH2 WCH3
Human IgG1 CH2-CH3
2H7 scFv
CH3
CH2
hulgAhinge
$V_L$
$V_H$
N434
H433
P331 / P331
K322
K320
E318/E318
H310
**N297/ **N297
I253
P238
G237
G236
L235
L234
E233
PASPSPTPPTSPSPTPPTSPVPQD
=ADCC and FcR binding
=Complement Fixation

FIG.12

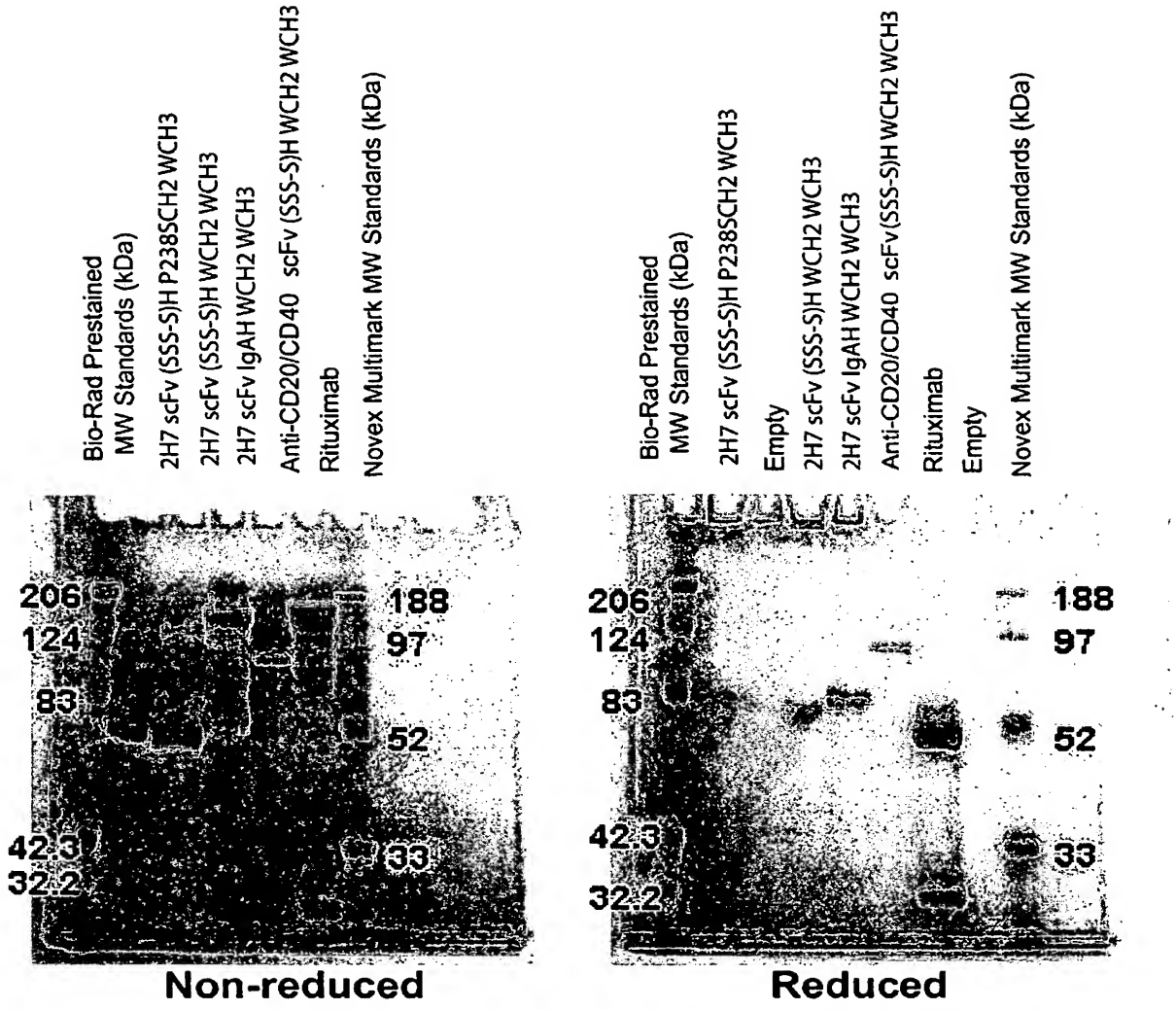

Bio-Rad Prestained MW Standards (kDa)
2H7 scFv (SSS-S)H P238SCH2 WCH3
2H7 scFv (SSS-S)H WCH2 WCH3
2H7 scFv IgAH WCH2 WCH3
Anti-CD20/CD40 scFv (SSS-S)H WCH2 WCH3
Rituximab
Novex Multimark MW Standards (kDa)
206
124
83
42.3
32.2
188
97
52
33
Non-reduced
Bio-Rad Prestained MW Standards (kDa)
2H7 scFv (SSS-S)H P238SCH2 WCH3
Empty
2H7 scFv (SSS-S)H WCH2 WCH3
2H7 scFv IgAH WCH2 WCH3
Anti-CD20/CD40 scFv (SSS-S)H WCH2 WCH3
Rituximab
Empty
Novex Multimark MW Standards (kDa)
206
124
83
42.3
32.2
188
97
52
33
Reduced

FIG.13

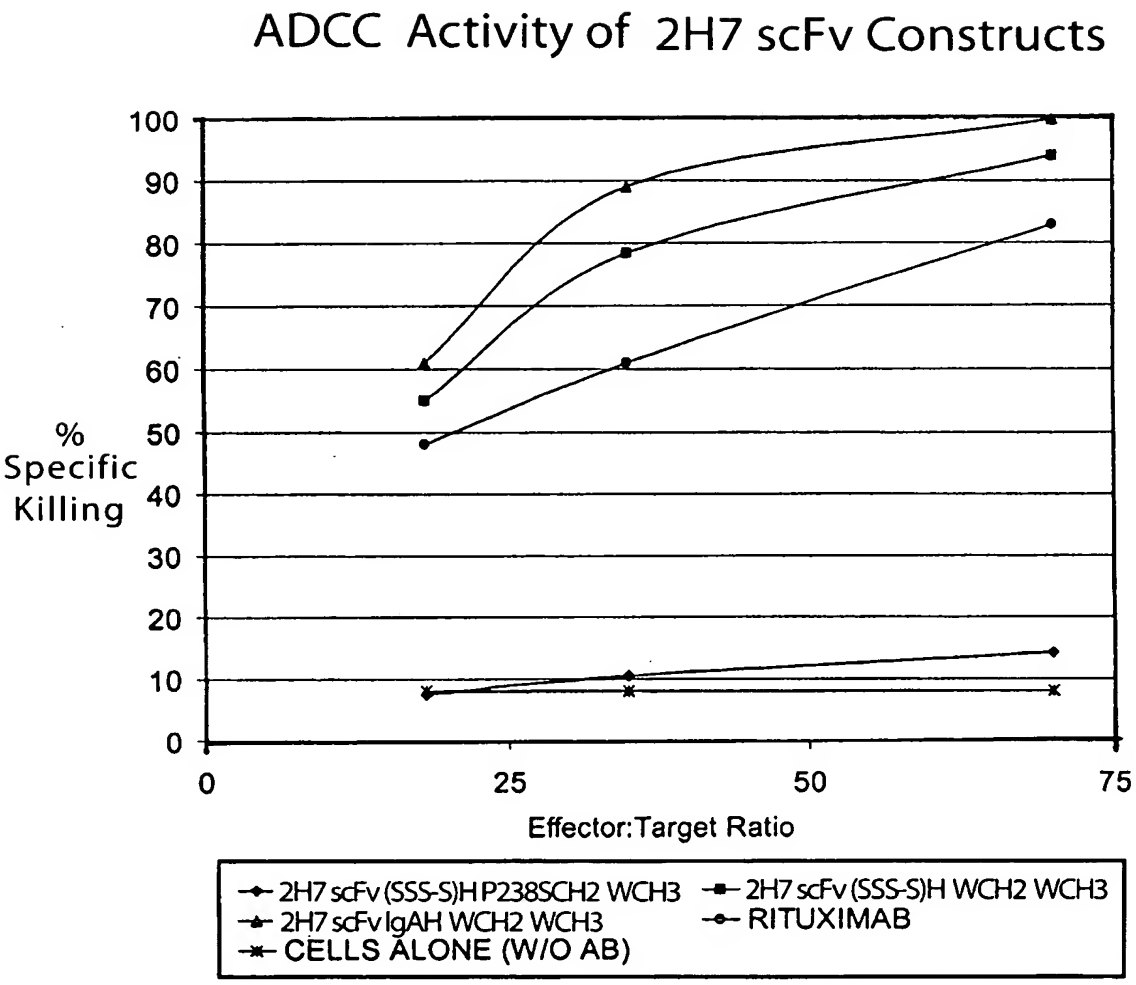
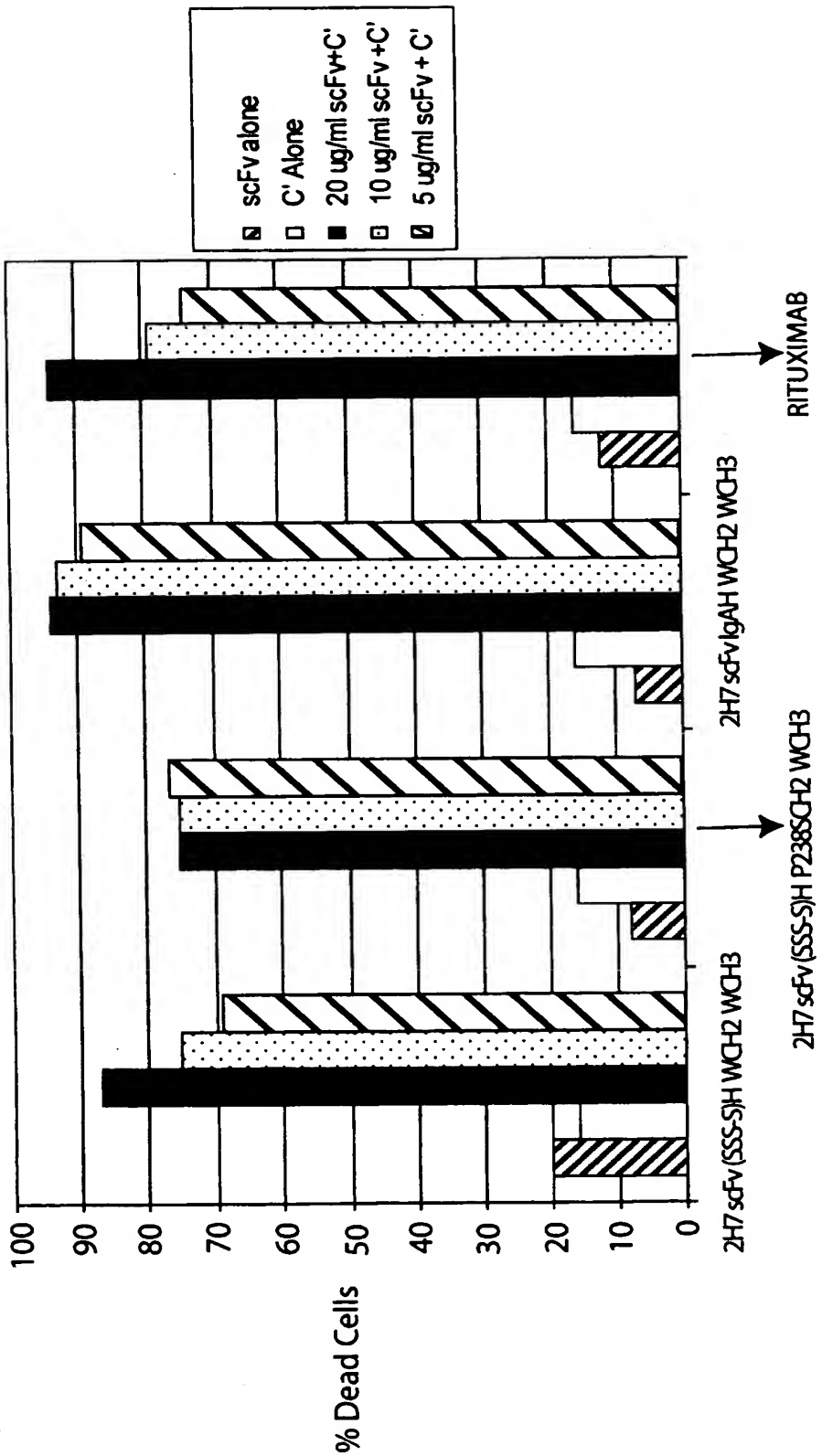
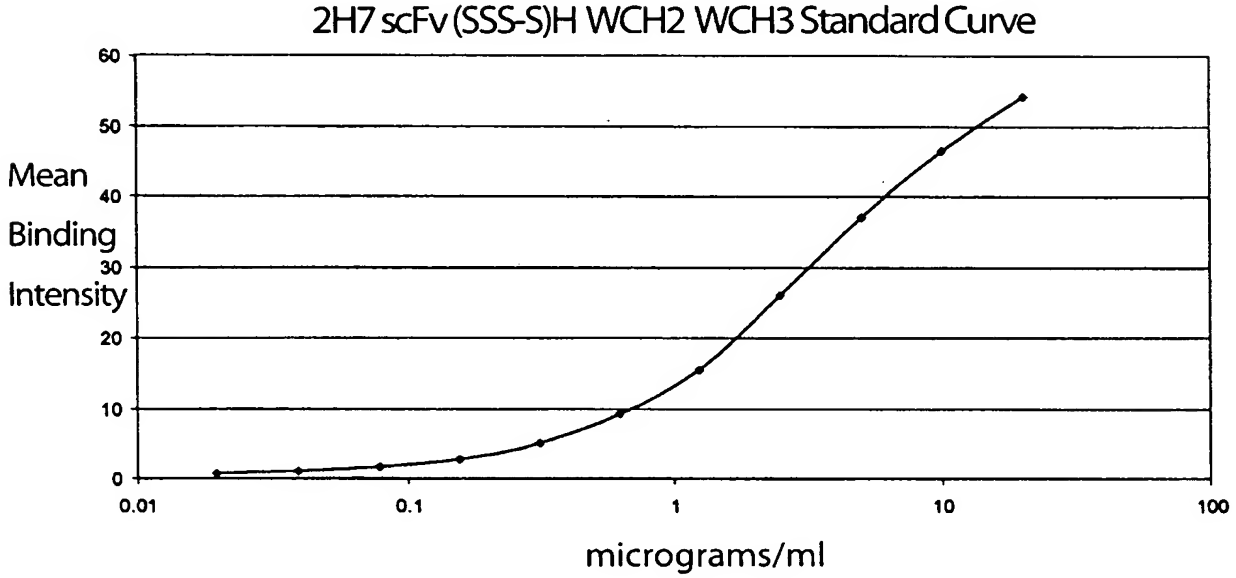

ADCC Activity of 2H7 scFv Constructs
% Specific Killing
100
90
80
70
60
50
40
30
20
10
0
0
25
50
75
Effector:Target Ratio
2H7 scFv (SSS-S)H P238SCH2 WCH3
2H7 scFv (SSS-S)H WCH2 WCH3
2H7 scFv IgAH WCH2 WCH3
RITUXIMAB
CELLS ALONE (W/O AB)

FIG. 14


scFv alone
C' Alone
20 ug/ml scFv+C'
10 ug/ml scFv +C'
5 ug/ml scFv + C'
% Dead Cells
100
90
80
70
60
50
40
30
20
10
0
2H7 scFv (SSS-S)H WCH2 WCH3
2H7 scFv (SSS-S)H P238SCH2 WCH3
2H7 scFv IgAH WCH2 WCH3
RITUXIMAB
Antibody Stimulus

# FIG. 15

## 2H7 scFv (SSS-S)H WCH2 WCH3 In Vivo Half Life



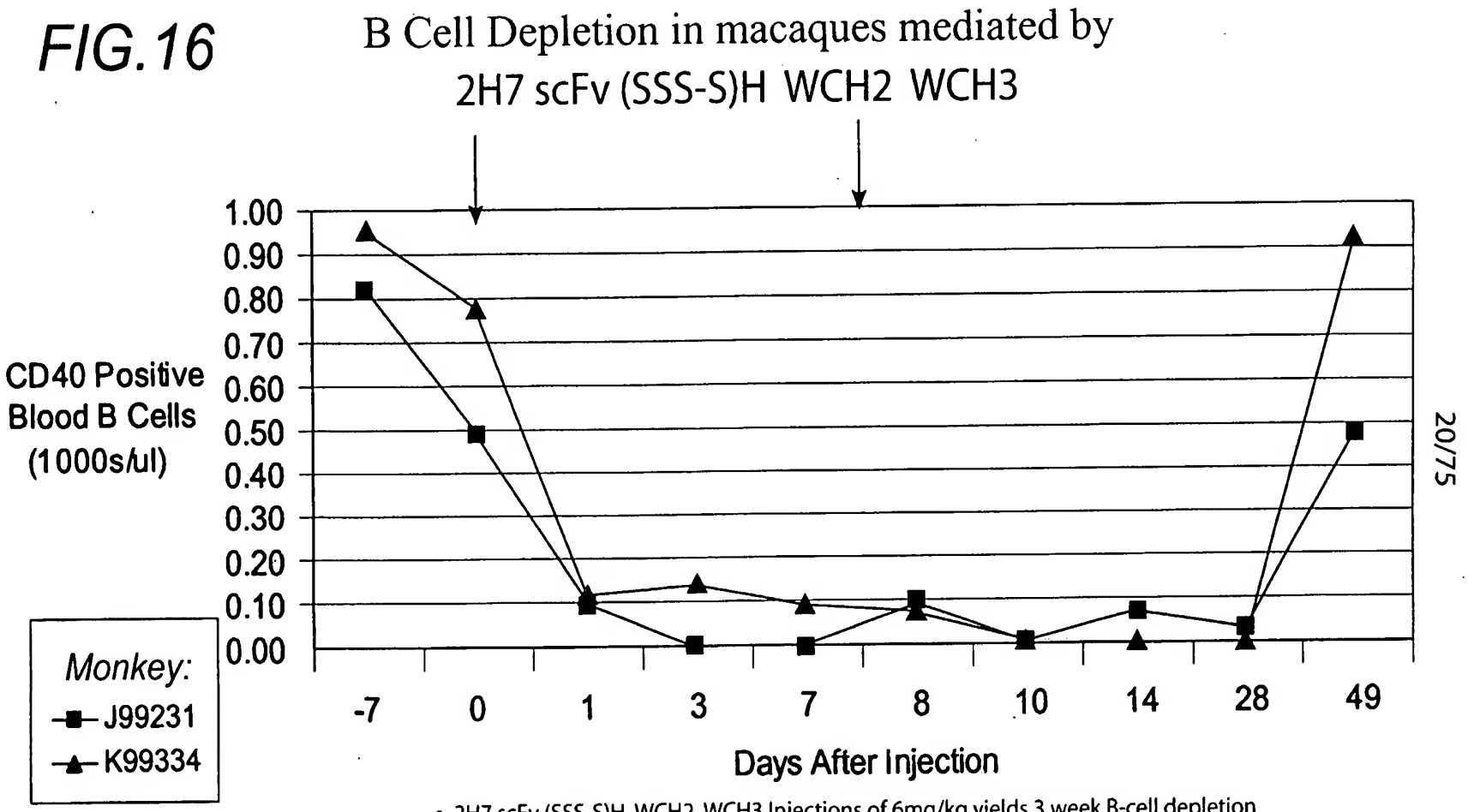

Macaque A99314

| | Day | Binding intensity At 1:50 | estimated concentration (μg/ml) |
|---|---|---|---|
| | –7 | 0.213 | <0.1 |
| Injection #1 → | 0 | 0.227 | <0.1 |
| | 1 | 7.79 | 25.1 |
| | 3 | 5.51 | 15.6 |
| | 7 | 3.37 | 9.4 |
| Injection #2 → | 8 | 11.33 | 41.7 |
| | 10 | 5.45 | 15.4 |
| | 14 | 0.27 | <0.1 |

Macaque F98081

| | Day | Binding intensity At 1:50 | estimated concentration (μg/ml) |
|---|---|---|---|
| | –7 | 0.208 | <0.1 |
| Injection #1 → | 0 | 0.219 | <0.1 |
| | 1 | 6.73 | 21.9 |
| | 3 | 6.14 | 19.3 |
| | 7 | 3.04 | 8.7 |
| Injection #2 → | 8 | 9.83 | 33.8 |
| | 10 | 4.77 | 14.4 |
| | 14 | 0.231 | <0.1 |

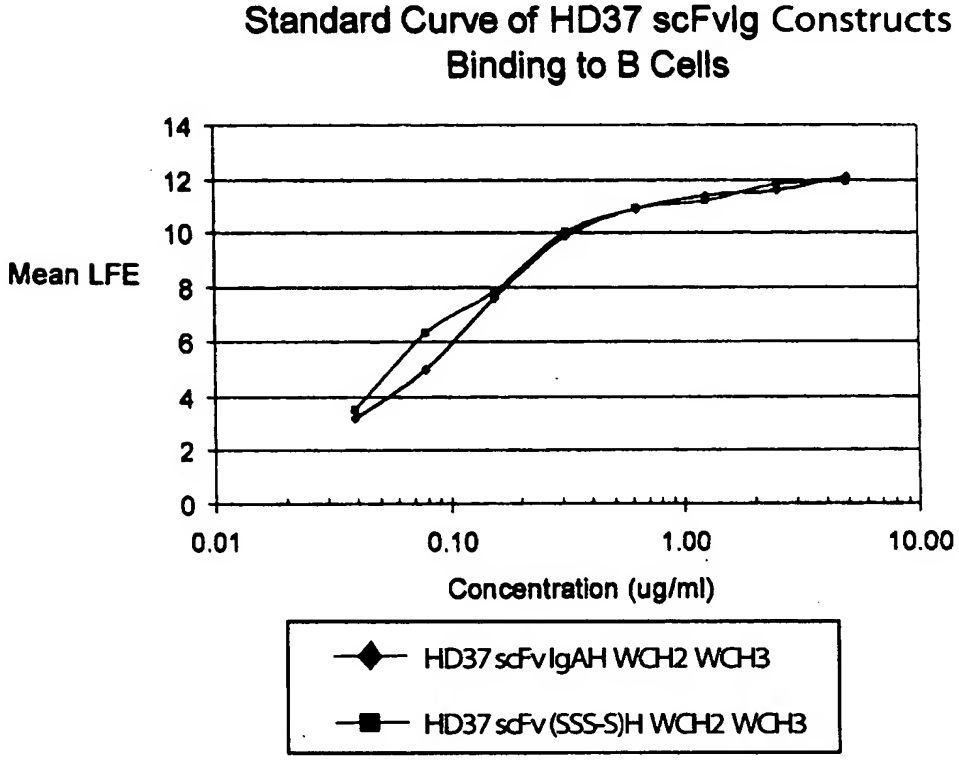
FIG.16




- 2H7 scFv (SSS-S)H WCH2 WCH3 Injections of 6mg/kg yields 3 week B-cell depletion
- **3-4 day half-life *in vivo***
- **CD20 saturation in lymph node B-cells at d14**
- **No first dose effects**
- **No anti-chimeric antibody development**

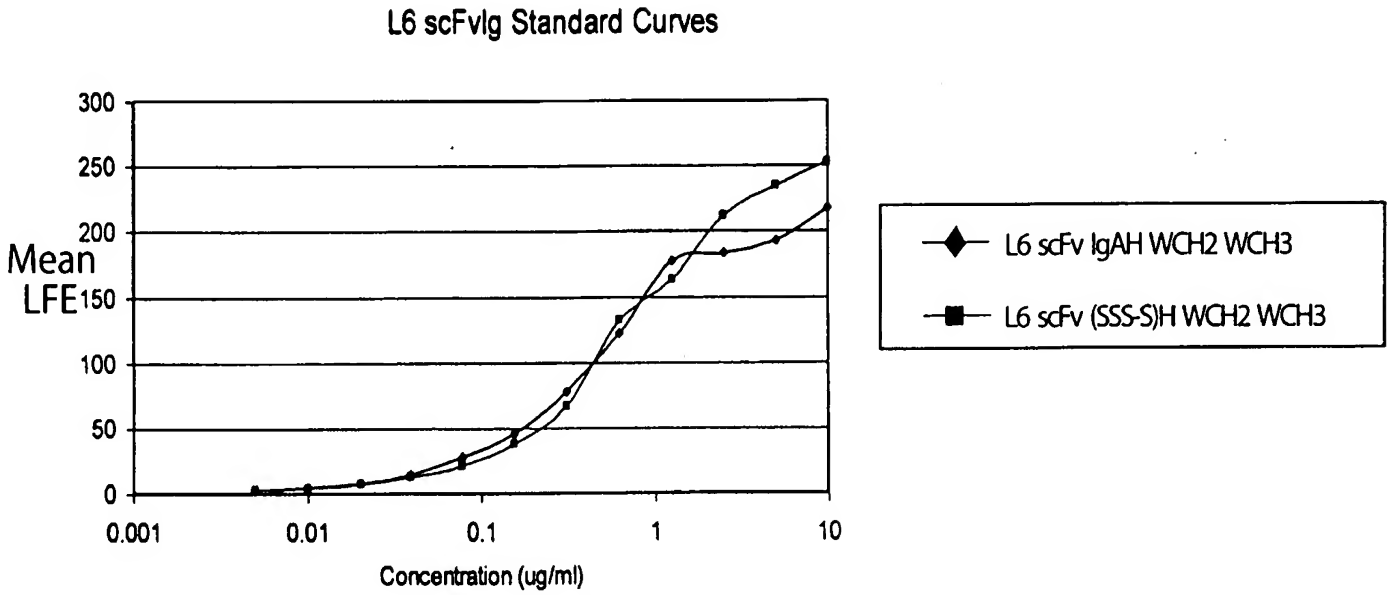
# FIG. 17

## Production Levels of HD37 scFv Constructs by CHO Cell Lines




| Clone/Isolate | Mean LFE at 1:100 | Estimated Concentration |
|---|---|---|
| HD37 scFv IgAH WCH2 WCH3 | 11.2 | > 60 ug/ml |
| 1B2 | 10.4 | >50 ug/ml |
| 6C5 | 10.5 | >50 ug/ml |
| 4B1 | 8.6 | >40 ug/ml |
| | | |
| HD37 scFv (SSS-S)H WCH2 WCH3 | 10.9 | > 50 ug/ml |
| 2G8 | 10.6 | > 50 ug/ml |
| 3F3 | 8.3 | >40 ug/ml |
| 3D9 | 11.1 | > 60 ug/ml |

# FIG.18 Production of L6 scFvIg Constructs by CHO Cells




| Construct | Mean LFE 1:20 | Estimated Concentration |
|---|---|---|
| L6 scFv IgAH WCH2 WCH3 unamplified CHO sup | 51.1 | 6.25 ug/ml |
| L6 scFv (SSS-S)H WCH2 WCH3 unamplified CHO sup | 23.0 | 3.2 ug/ml |

FIG. 19A

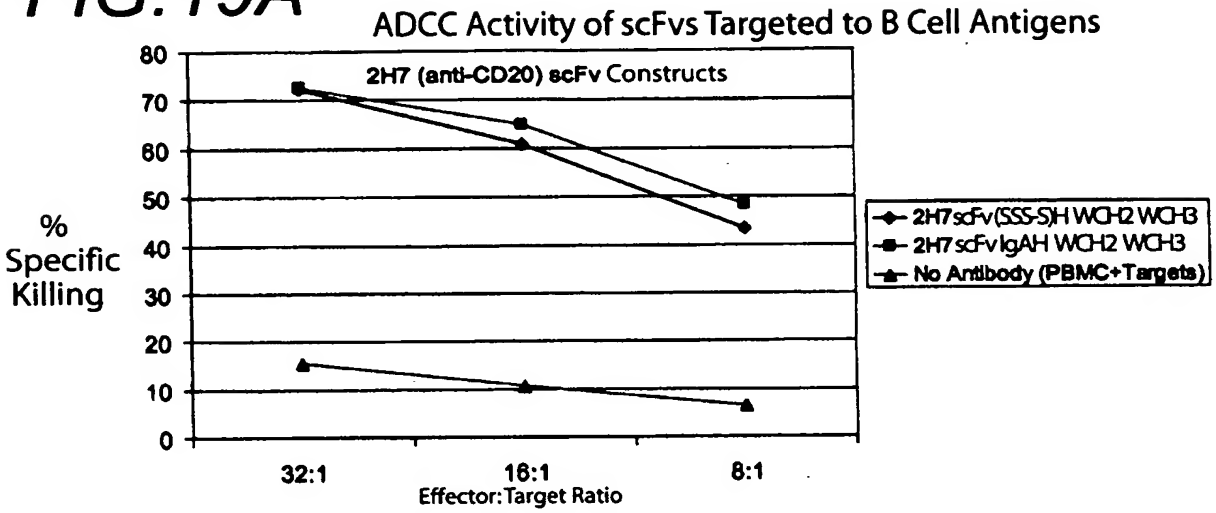



FIG. 19B

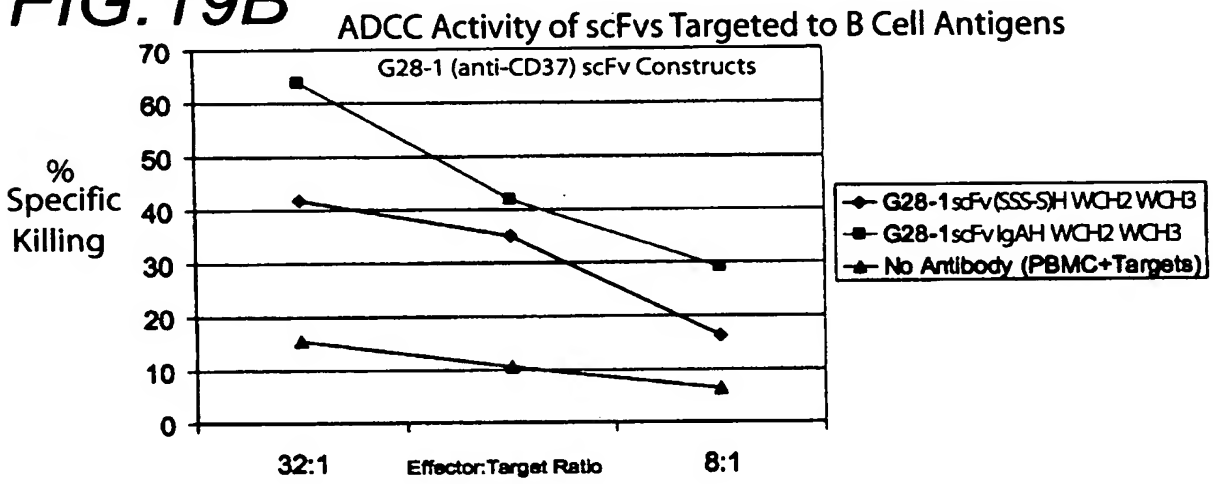



FIG. 19C

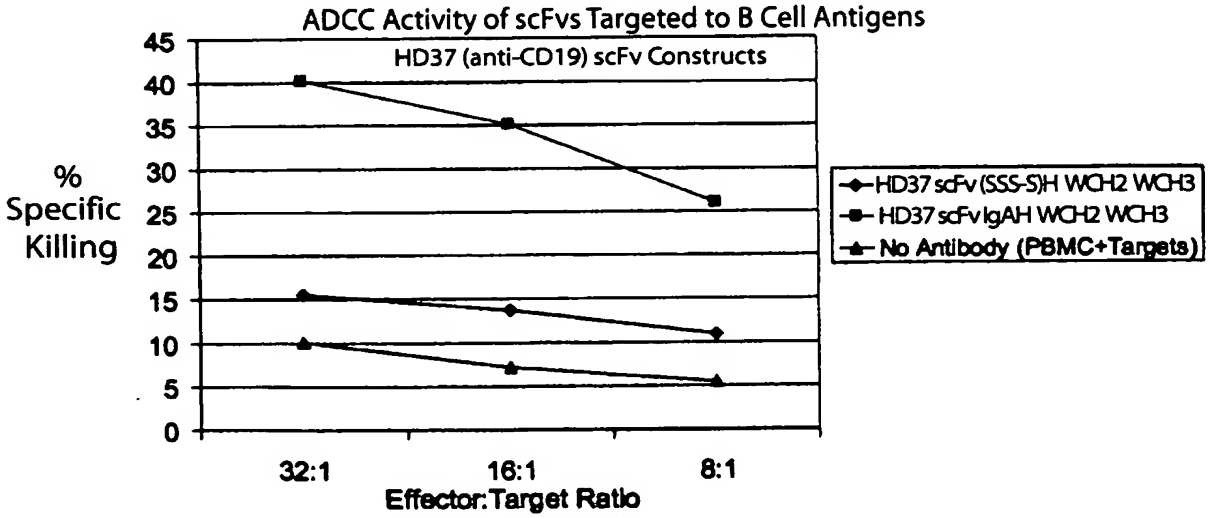


FIG.20

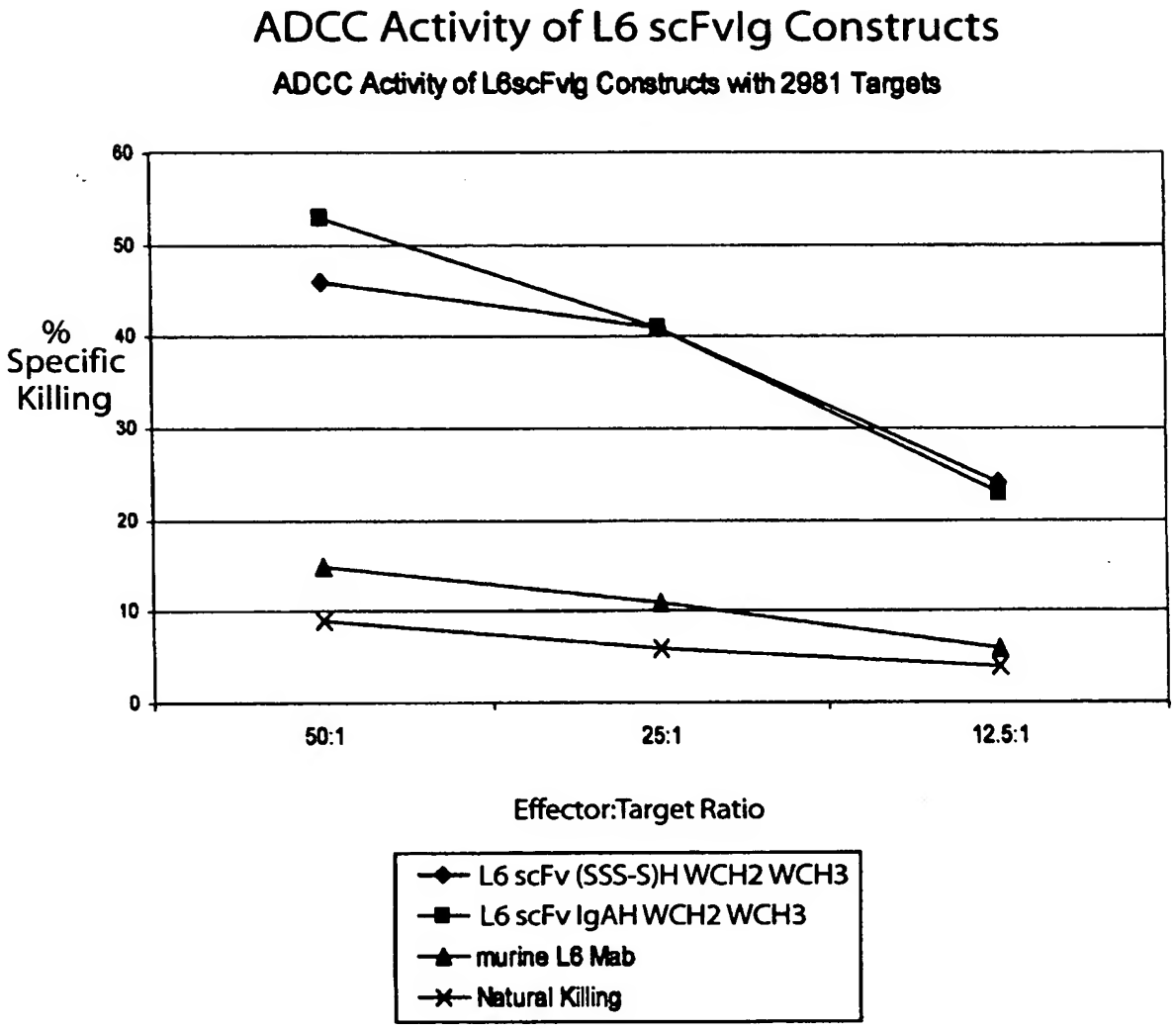

ADCC Activity of L6 scFvIg Constructs
ADCC Activity of L6scFvIg Constructs with 2981 Targets
% Specific Killing
60
50
40
30
20
10
0
50:1
25:1
12.5:1
Effector:Target Ratio
L6 scFv (SSS-S)H WCH2 WCH3
L6 scFv IgAH WCH2 WCH3
murine L6 Mab
Natural Killing

FIG.21

SDS-PAGE Analysis of L6 and 2H7 scFvIg Constructs

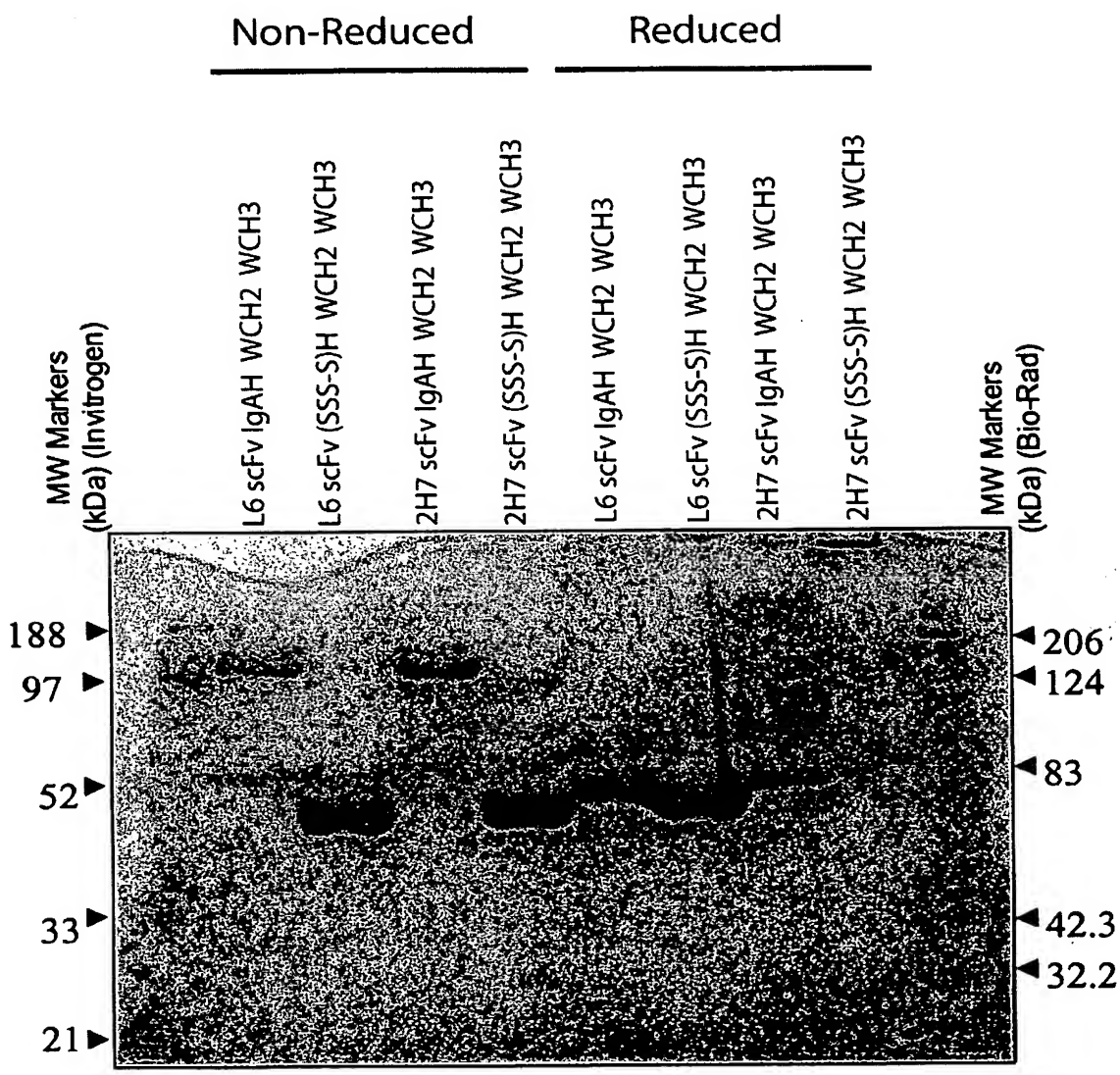
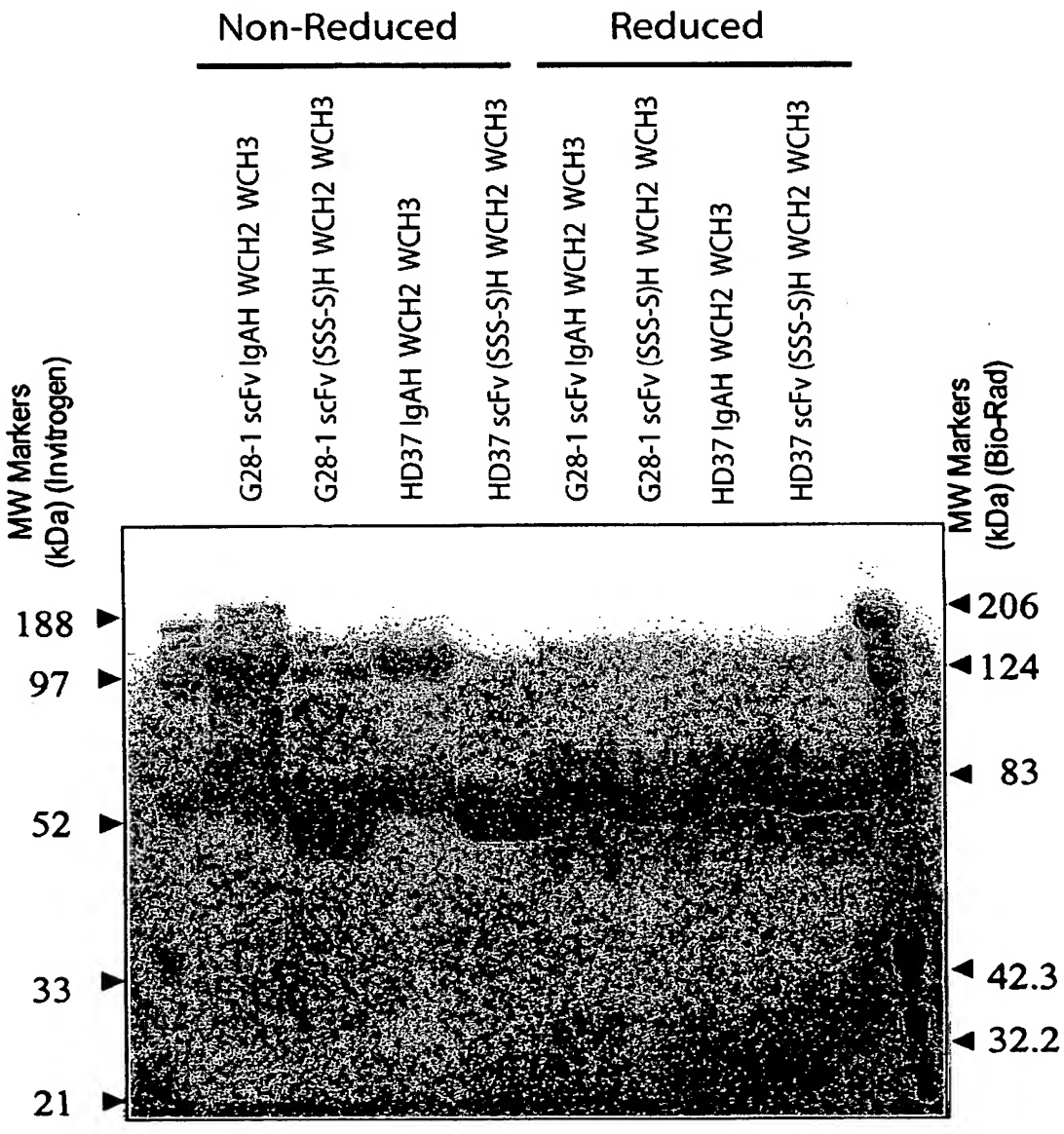


FIG.22

SDS-PAGE Analysis of G28-1 and HD37 scFvIg Constructs



# FIG.23

Sequence alignment of human and Llama Fc regins.

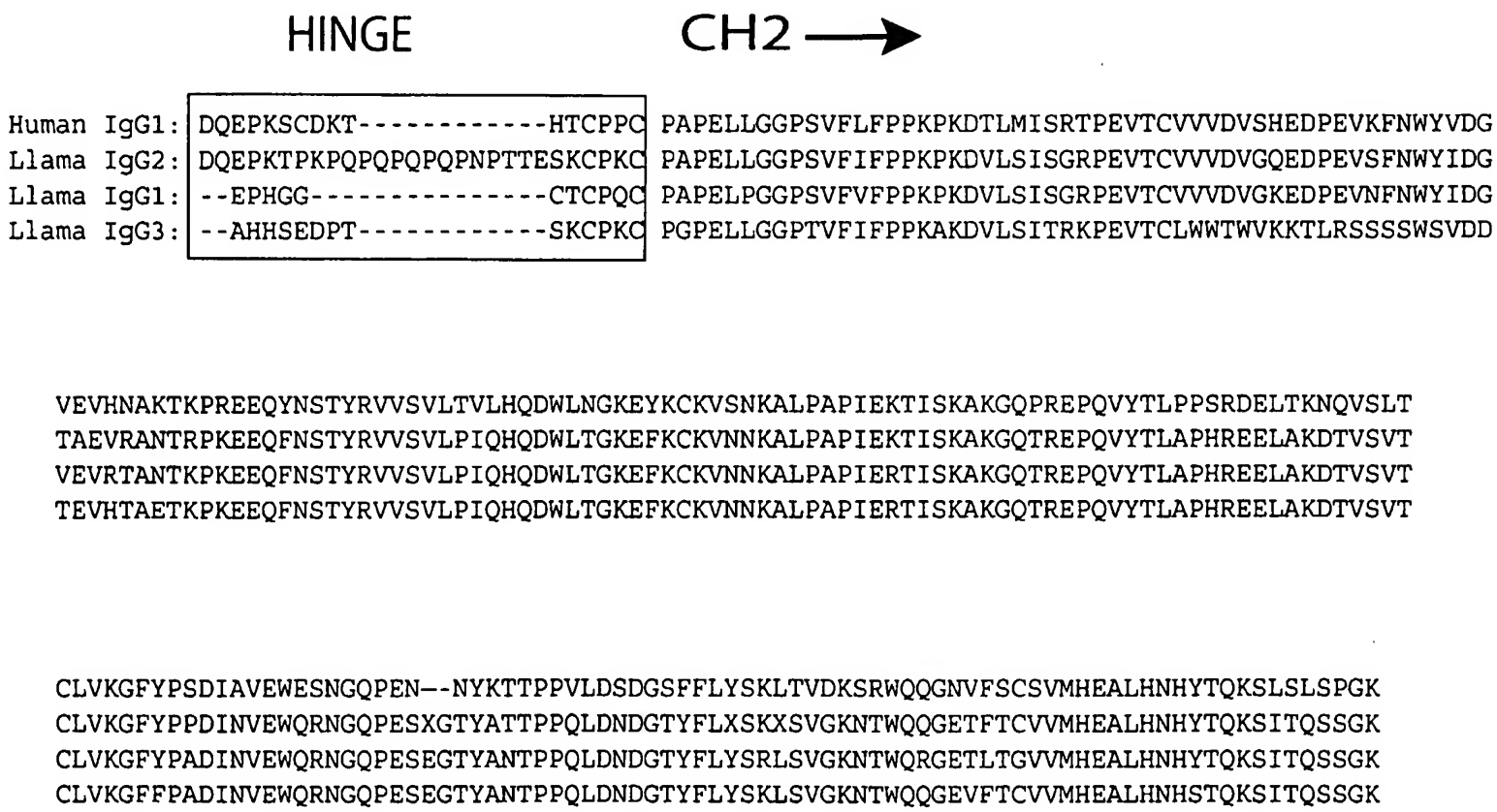


FIG.24

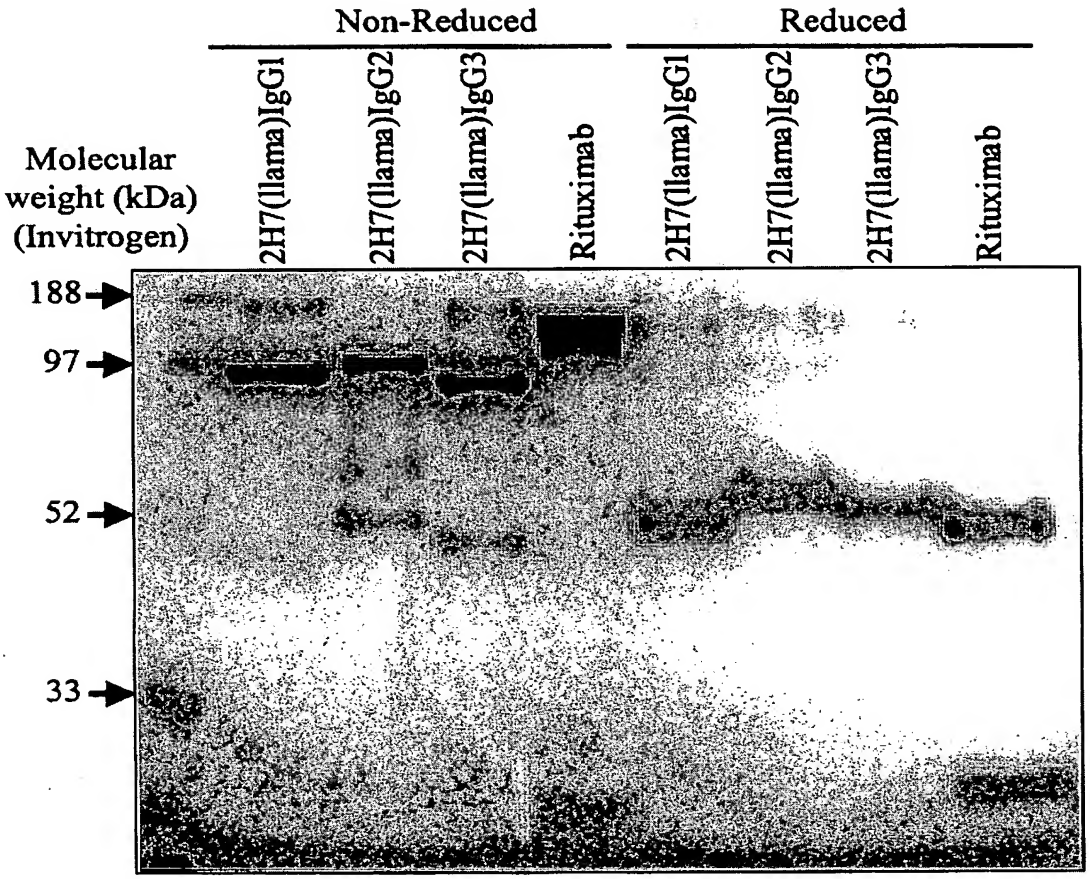

Non-Reduced
Reduced
2H7(llama)IgG1
2H7(llama)IgG2
2H7(llama)IgG3
Rituximab
2H7(llama)IgG1
2H7(llama)IgG2
2H7(llama)IgG3
Rituximab
Molecular weight (kDa) (Invitrogen)
188
97
52
33

FIG.25

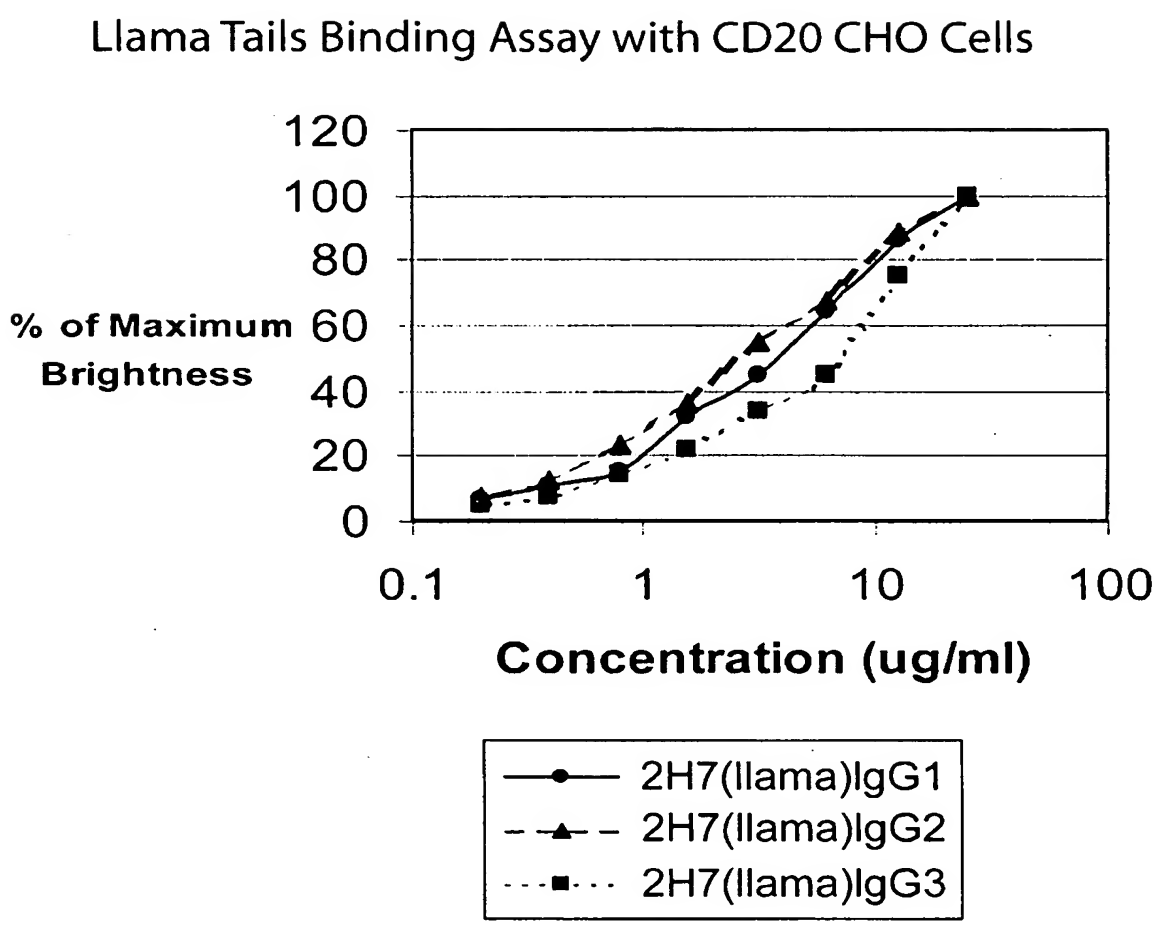

Llama Tails Binding Assay with CD20 CHO Cells
% of Maximum Brightness
120
100
80
60
40
20
0
0.1
1
10
100
Concentration (ug/ml)
2H7(llama)IgG1
2H7(llama)IgG2
2H7(llama)IgG3

FIG.26

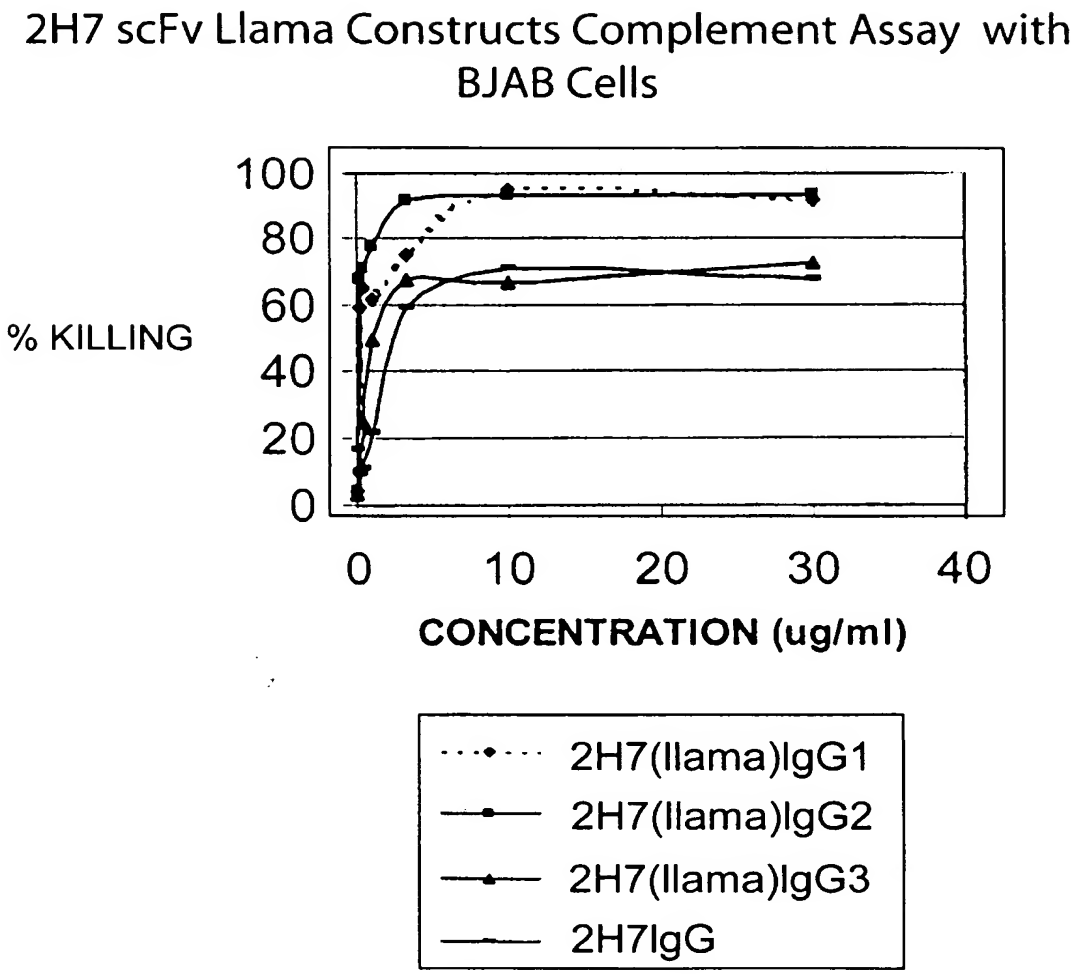

2H7 scFv Llama Constructs Complement Assay with BJAB Cells
% KILLING
100
80
60
40
20
0
0
10
20
30
40
CONCENTRATION (ug/ml)
2H7(llama)IgG1
2H7(llama)IgG2
2H7(llama)IgG3
2H7IgG

FIG.27

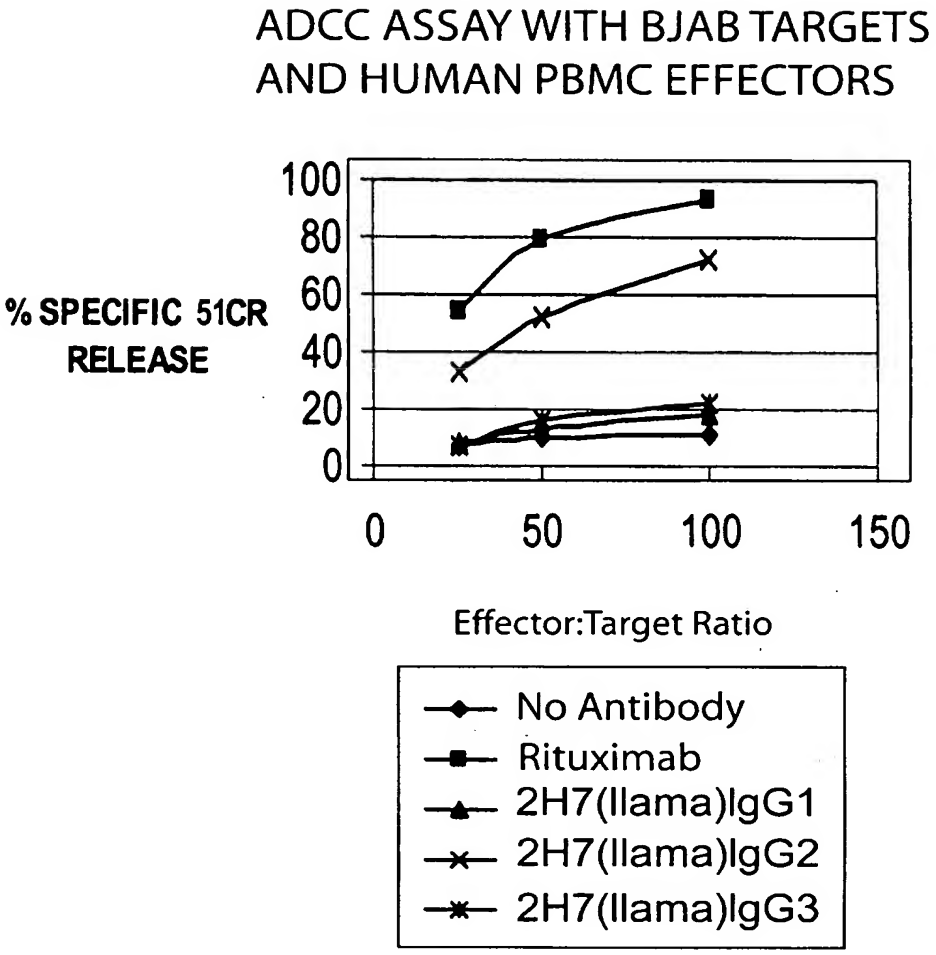

ADCC ASSAY WITH BJAB TARGETS
AND HUMAN PBMC EFFECTORS
% SPECIFIC 51CR RELEASE
100
80
60
40
20
0
0
50
100
150
Effector:Target Ratio
No Antibody
Rituximab
2H7(llama)IgG1
2H7(llama)IgG2
2H7(llama)IgG3

FIG.28

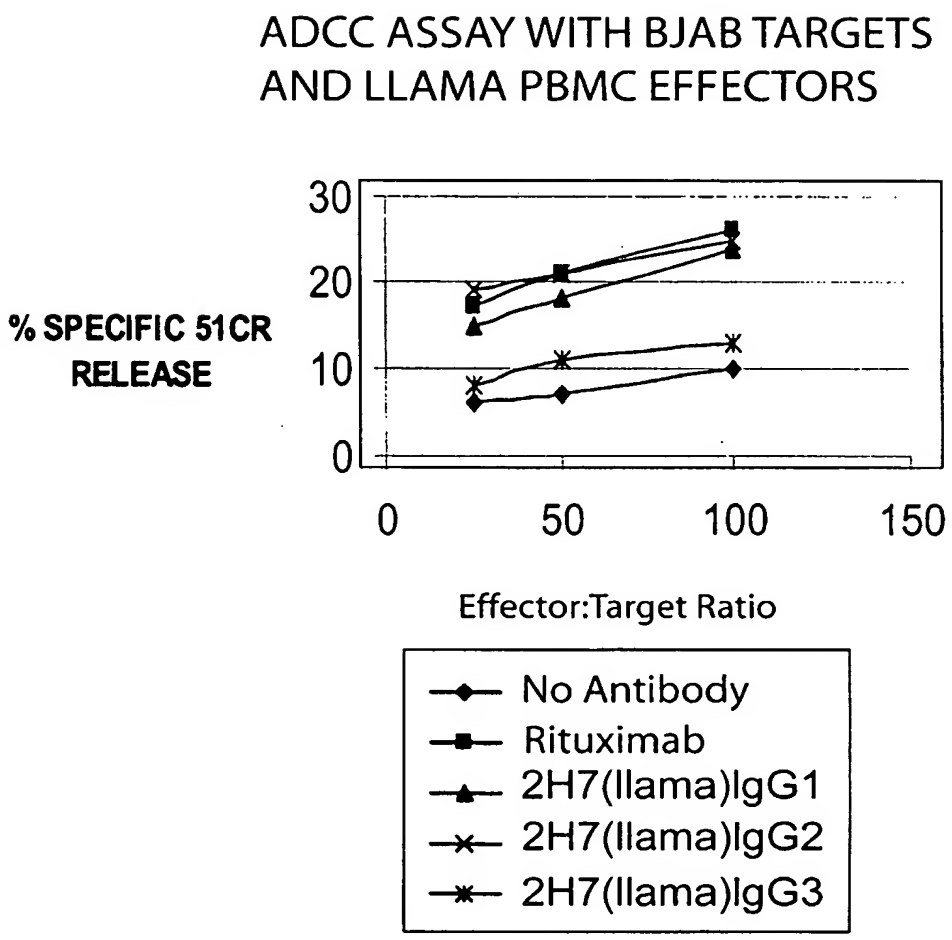

ADCC ASSAY WITH BJAB TARGETS
AND LLAMA PBMC EFFECTORS
% SPECIFIC 51CR RELEASE
30
20
10
0
0
50
100
150
Effector:Target Ratio
No Antibody
Rituximab
2H7(llama)IgG1
2H7(llama)IgG2
2H7(llama)IgG3

FIG.29

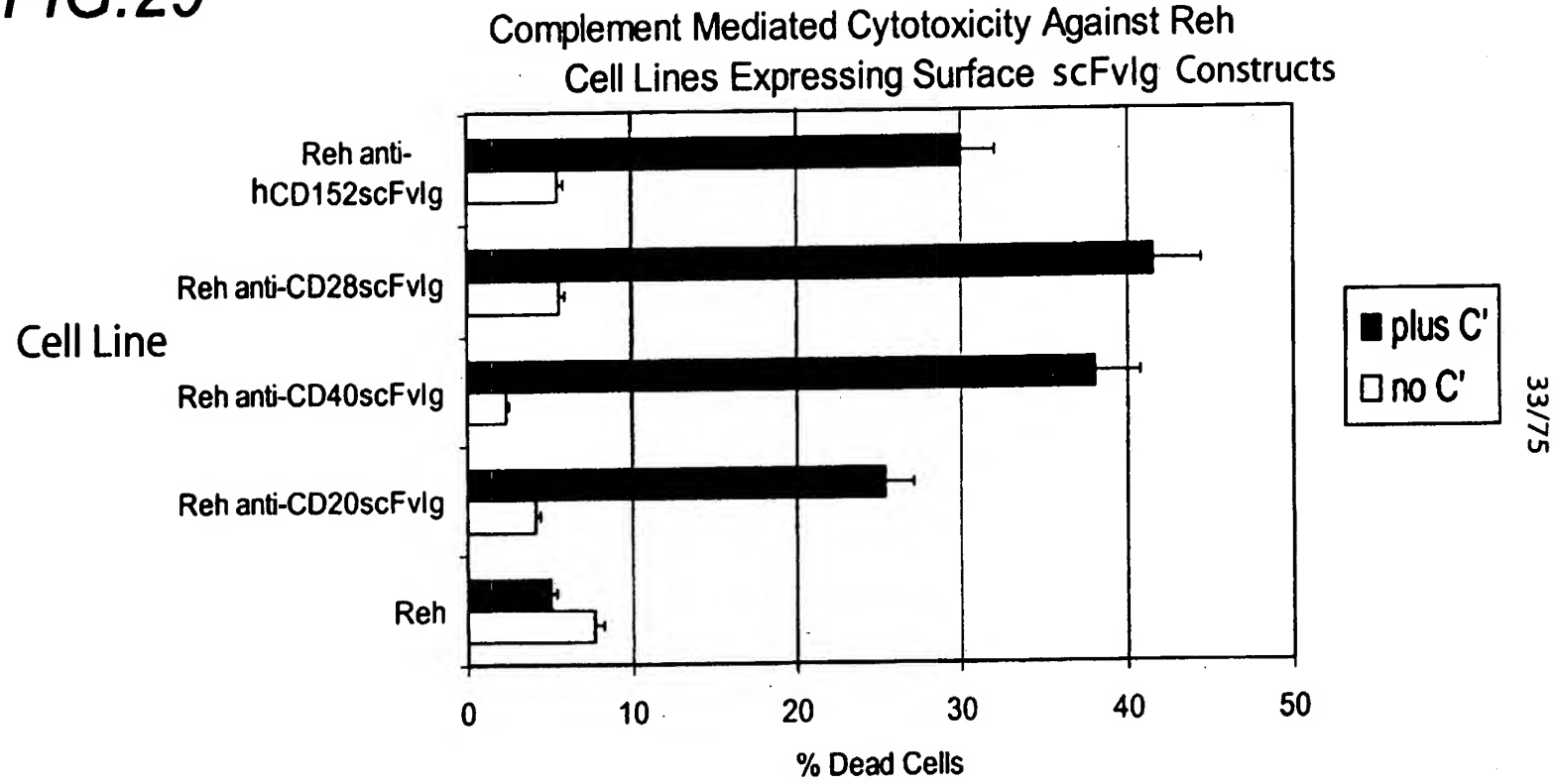

Complement Mediated Cytotoxicity Against Reh
Cell Lines Expressing Surface scFvIg Constructs
Cell Line
Reh anti-hCD152scFvIg
Reh anti-CD28scFvIg
Reh anti-CD40scFvIg
Reh anti-CD20scFvIg
Reh
plus C'
no C'
0
10
20
30
40
50
% Dead Cells

FIG.30

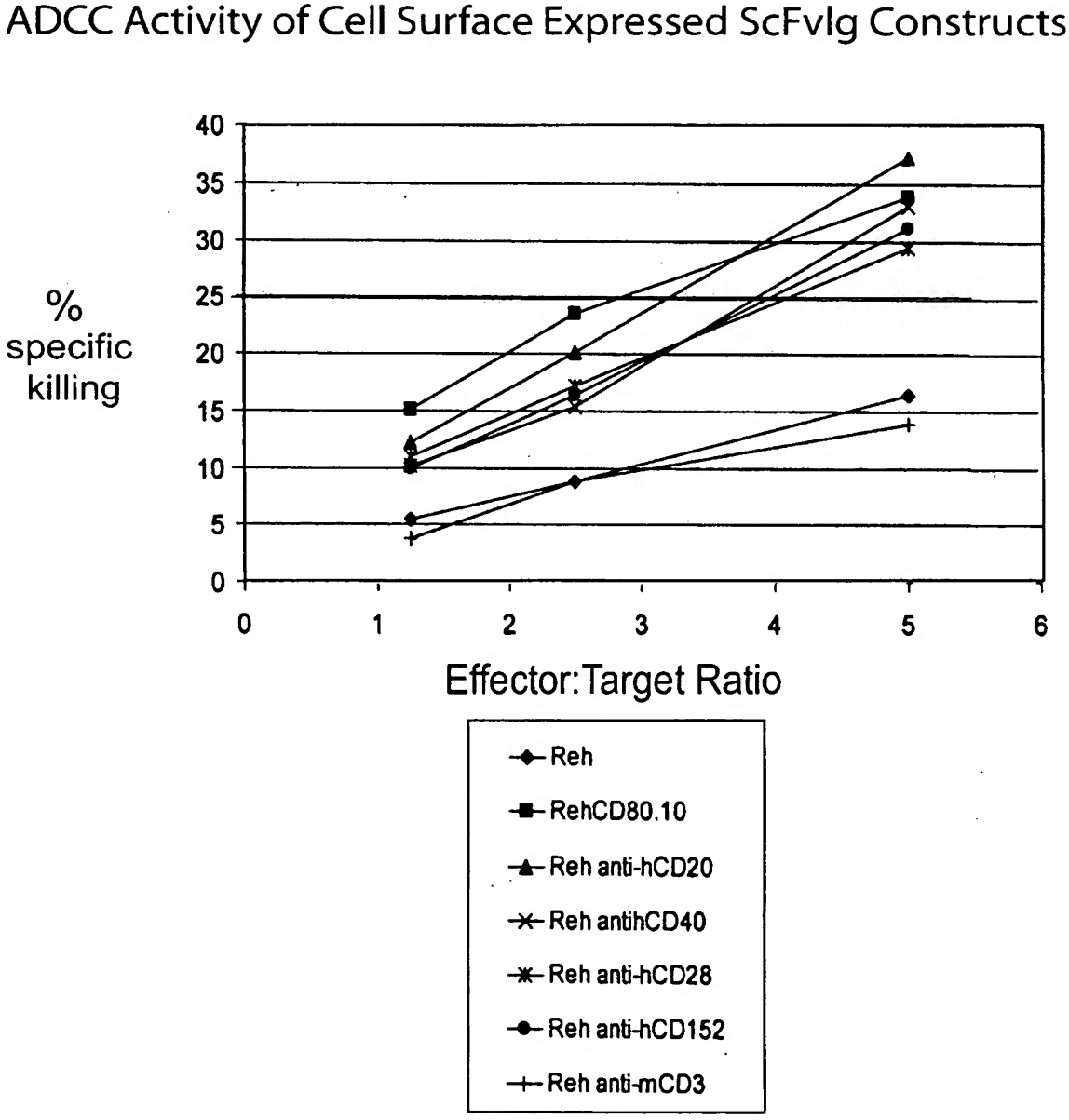

ADCC Activity of Cell Surface Expressed ScFvIg Constructs
% specific killing
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
Effector:Target Ratio
Reh
RehCD80.10
Reh anti-hCD20
Reh antihCD40
Reh anti-hCD28
Reh anti-hCD152
Reh anti-mCD3

FIG.31

# Ig Constructs and Nomenclature:

| Name Identifier | Hinge Sequence | CH2 Sequence | CH3 Sequence |
|---|---|---|---|
| (CCC-P)WH WCH2 WCH3 | IgG1 WT Hinge (CCC) | Wild Type CH2 | Wild Type CH3 |
| (SSS-S)H WCH2 WCH3 | IgG1 Mutant Hinge (SSS) | Wild type CH2 (IgG1) | Wild type CH3 (IgG1) |
| VHL11S (SSS-S)H WCH2 WCH3 | IgG1 Mutant Hinge (SSS) | Wild type CH2 (IgG1) | Wild type CH3 (IgG1) |
| (SSC-P)H WCH2 WCH3 | IgG1 Mutant Hinge (SSC) | Wild type CH2 (IgG1) | Wild type CH3 (IgG1) |
| (SCS-S)H WCH2 WCH3 | IgG1 Mutant Hinge (SCS) | Wild type CH2 (IgG1) | Wild type CH3 (IgG1) |
| (CSS-S)H WCH2 WCH3 | IgG1 Mutant Hinge (CSS) | Wild type CH2 (IgG1) | Wild type CH3 (IgG1) |
| (SSS-S)H P238SCH2WCH3 | IgG1 Mutant Hinge (SSS) | Mutant CH2 (IgG1) Pro→Ser 238 | Wild type CH3 (IgG1) |
| IgAH WCH2 WCH3 | IgA Hinge | Wild type CH2 (IgG1) | Wild type CH3 (IgG1) |
| IgAH IgA CH2CH3 | IgA Hinge | Wild type CH2 (IgA) | Wild type CH3 (IgA) |
| IgAH IgA CH2T4CH3 | IgA Hinge | Wild type CH2 (IgA) | Truncated CH3 (IgA) Missing 4 aa at COOH |

FIG.32

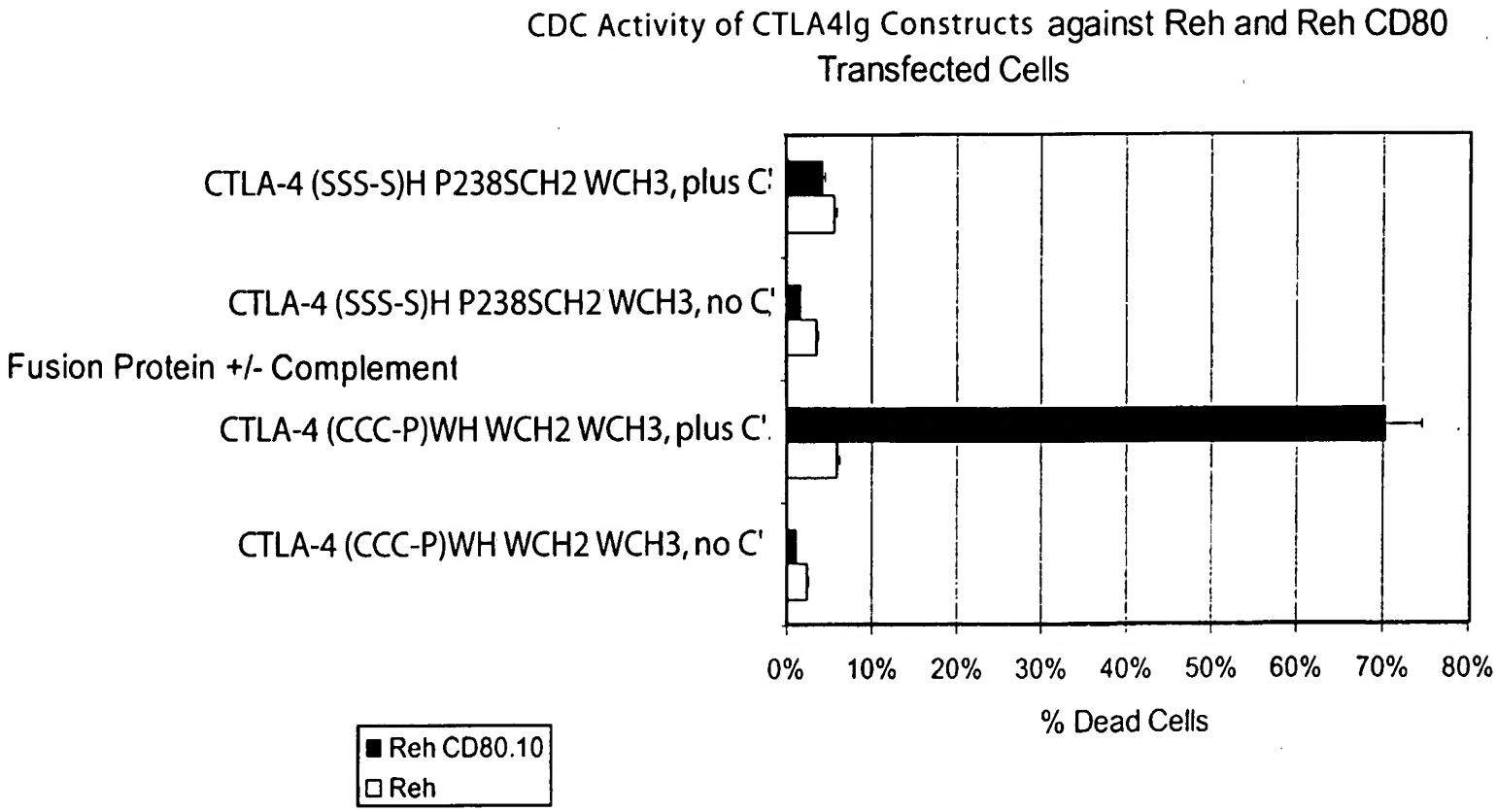

CDC Activity of CTLA4Ig Constructs against Reh and Reh CD80 Transfected Cells
CTLA-4 (SSS-S)H P238SCH2 WCH3, plus C'
CTLA-4 (SSS-S)H P238SCH2 WCH3, no C'
Fusion Protein +/- Complement
CTLA-4 (CCC-P)WH WCH2 WCH3, plus C'
CTLA-4 (CCC-P)WH WCH2 WCH3, no C'
0%
10%
20%
30%
40%
50%
60%
70%
80%
% Dead Cells
Reh CD80.10
Reh

FIG.33A

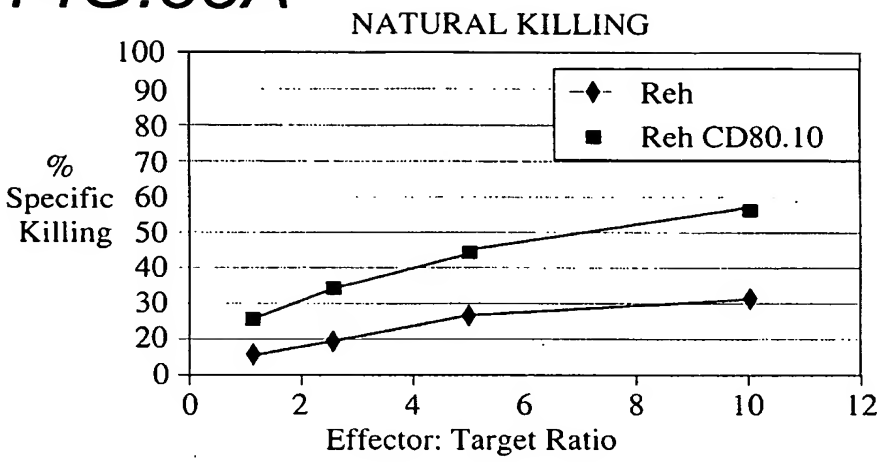



FIG.33B

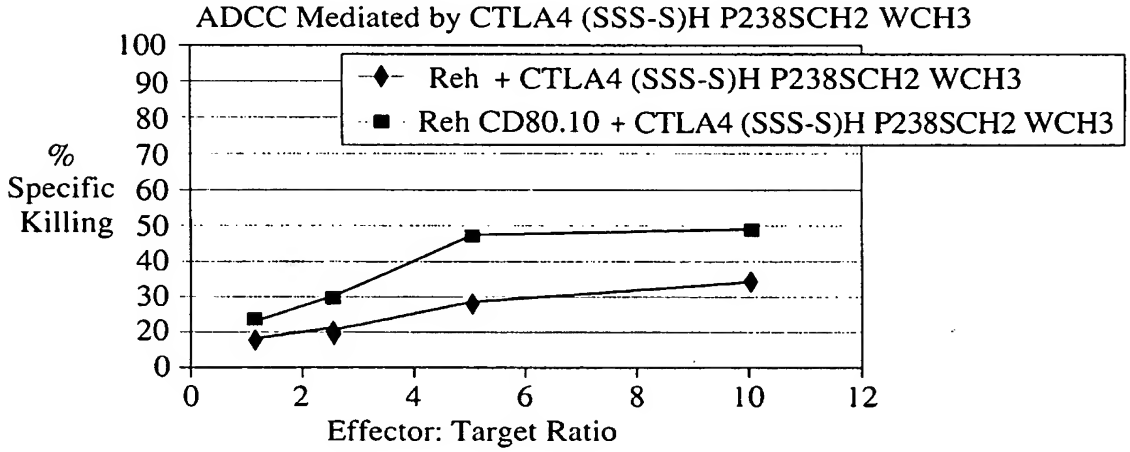



FIG.33C

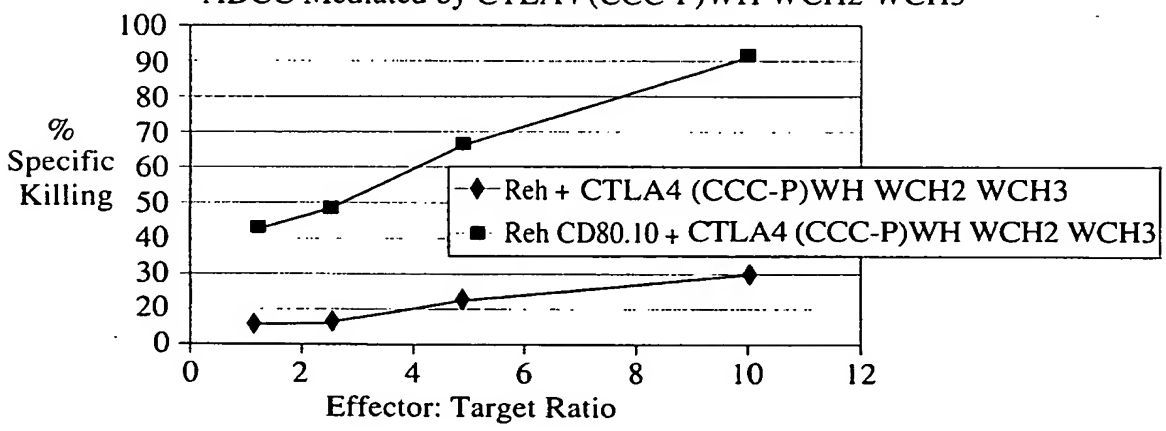


FIG.34

Binding of Constructs Alternative to CD2 CHO Cells

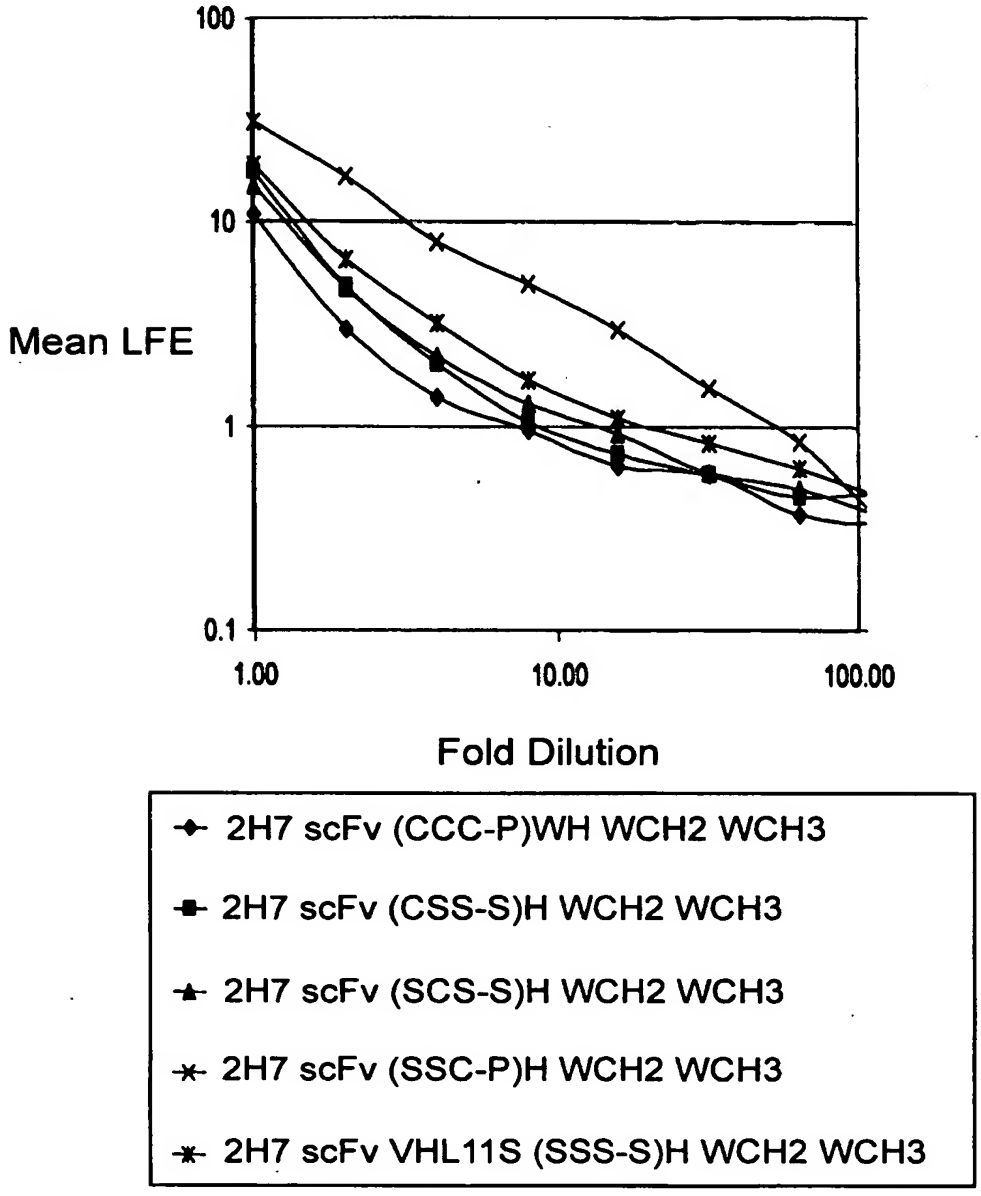


FIG.35

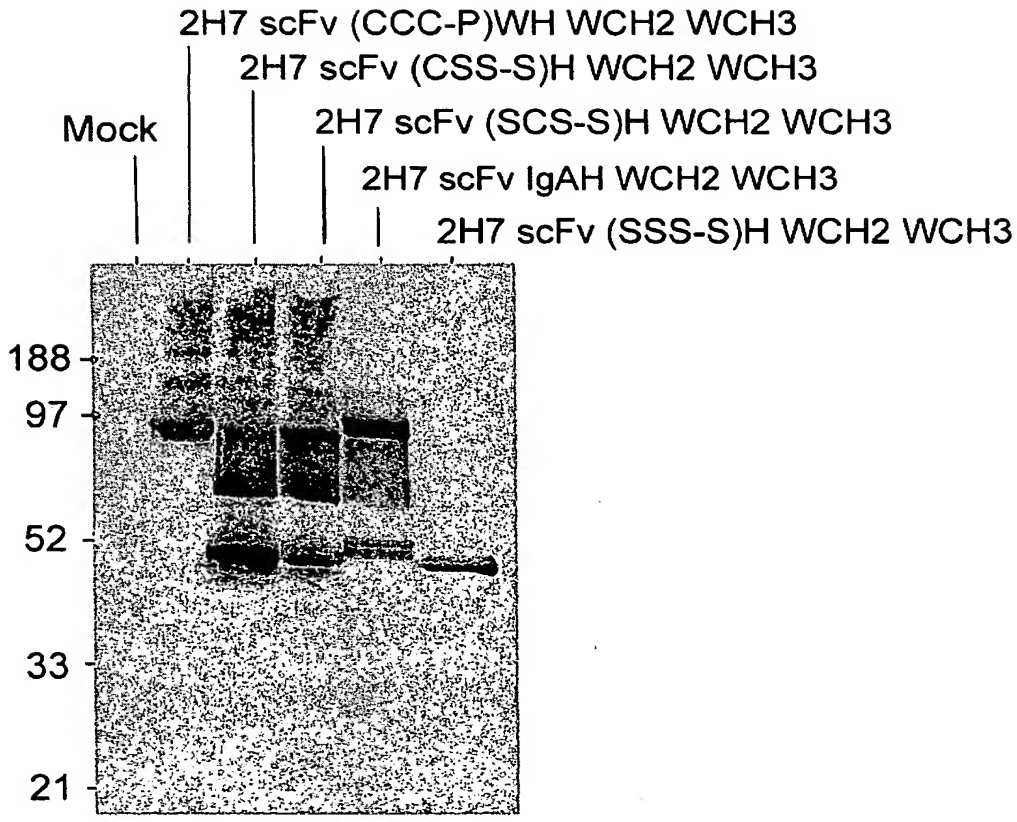

2H7 scFv (CCC-P)WH WCH2 WCH3
2H7 scFv (CSS-S)H WCH2 WCH3
Mock
2H7 scFv (SCS-S)H WCH2 WCH3
2H7 scFv IgAH WCH2 WCH3
2H7 scFv (SSS-S)H WCH2 WCH3
188
97
52
33
21

FIG.36

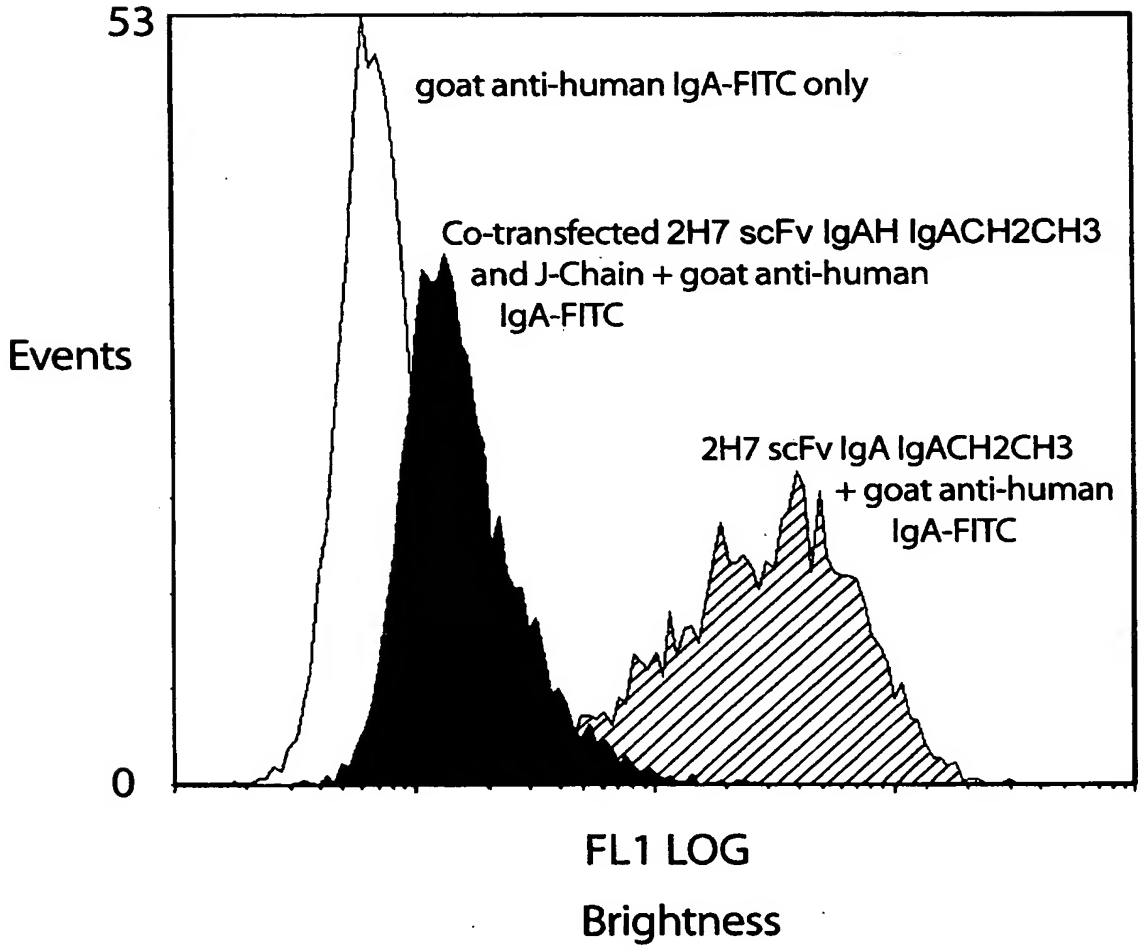

Binding to CD20 CHO cells by Constructs
that link anti-CD20 scFv to IgA Fc Domains
53
0
Events
goat anti-human IgA-FITC only
Co-transfected 2H7 scFv IgAH IgACH2CH3
and J-Chain + goat anti-human
IgA-FITC
2H7 scFv IgA IgACH2CH3
+ goat anti-human
IgA-FITC
FL1 LOG
Brightness

FIG.37

Titration of CD20 Specific scFvIg Constructs for ADCC Activity Using Whole Blood Effectors

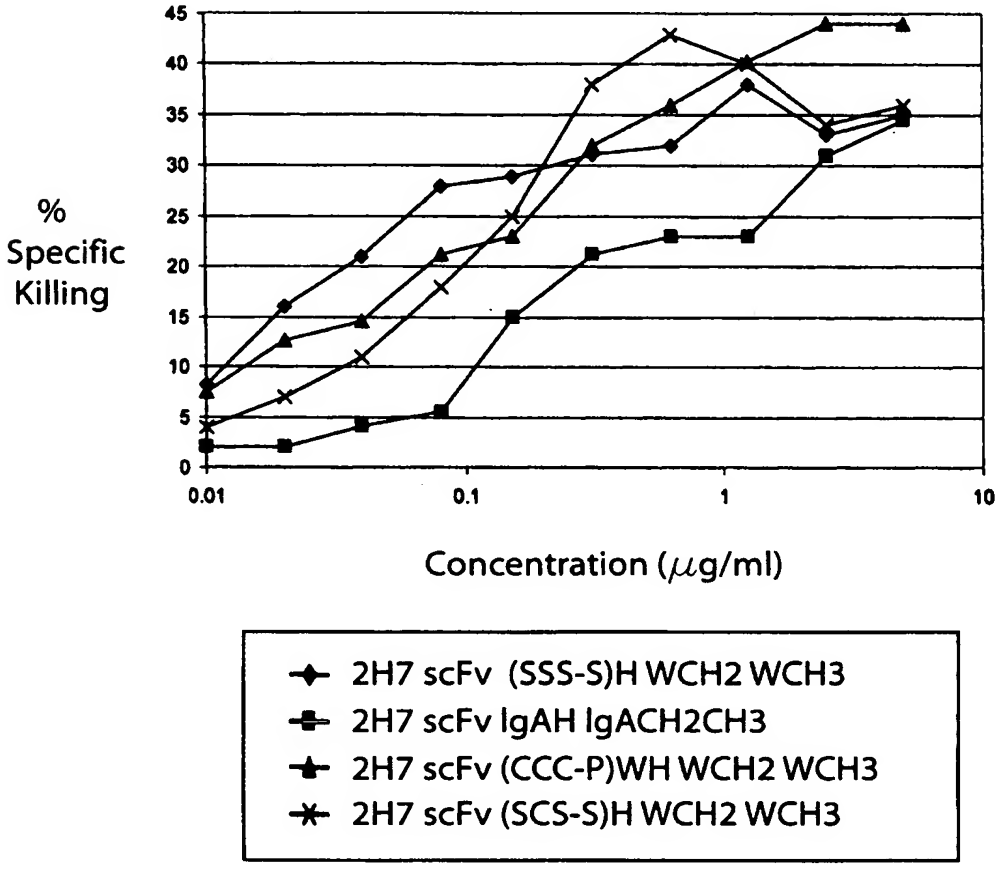


FIG.38

ADCC ASSAY OF ANTI-CD20 CONSTRUCTS WITH DIFFERENT TAILS
(WHOLE BLOOD EFFECTORS/BJAB TARGETS)

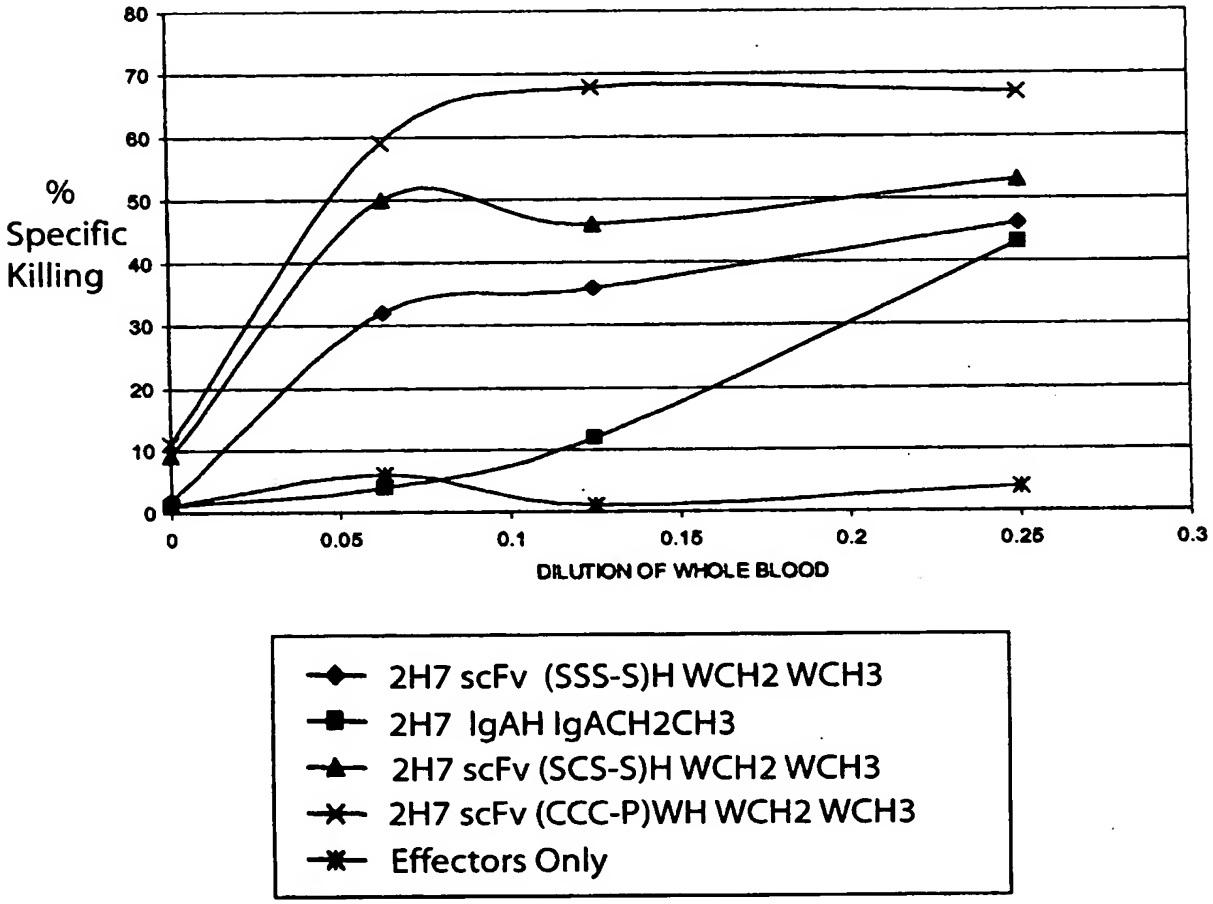


FIG.39A

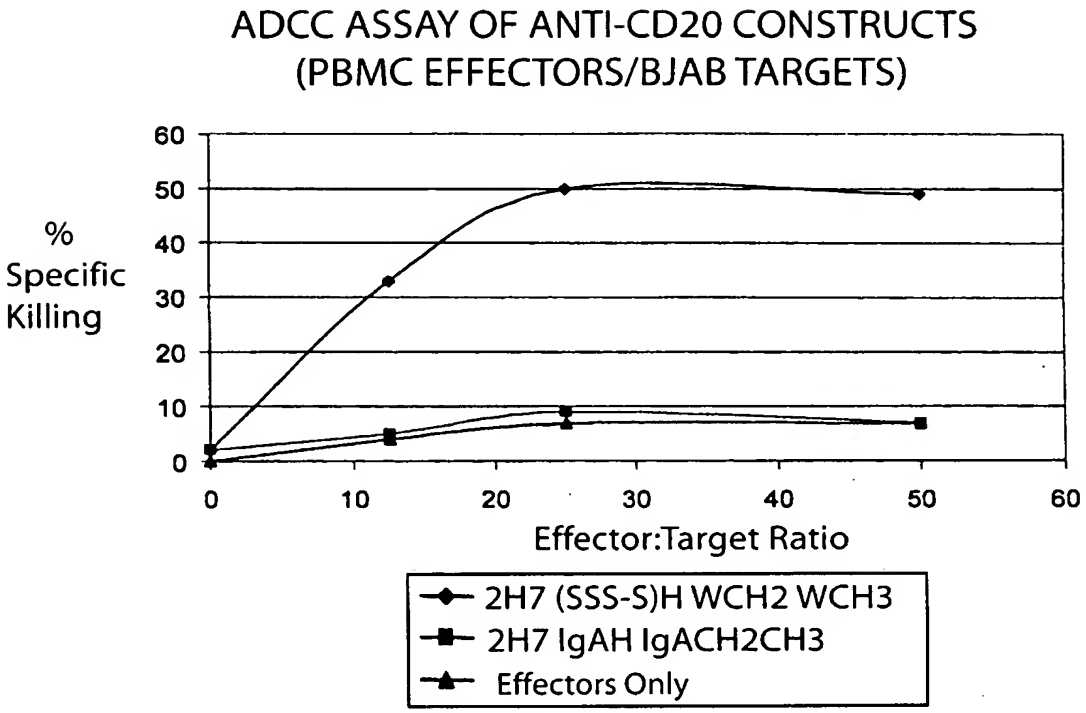



FIG.39B

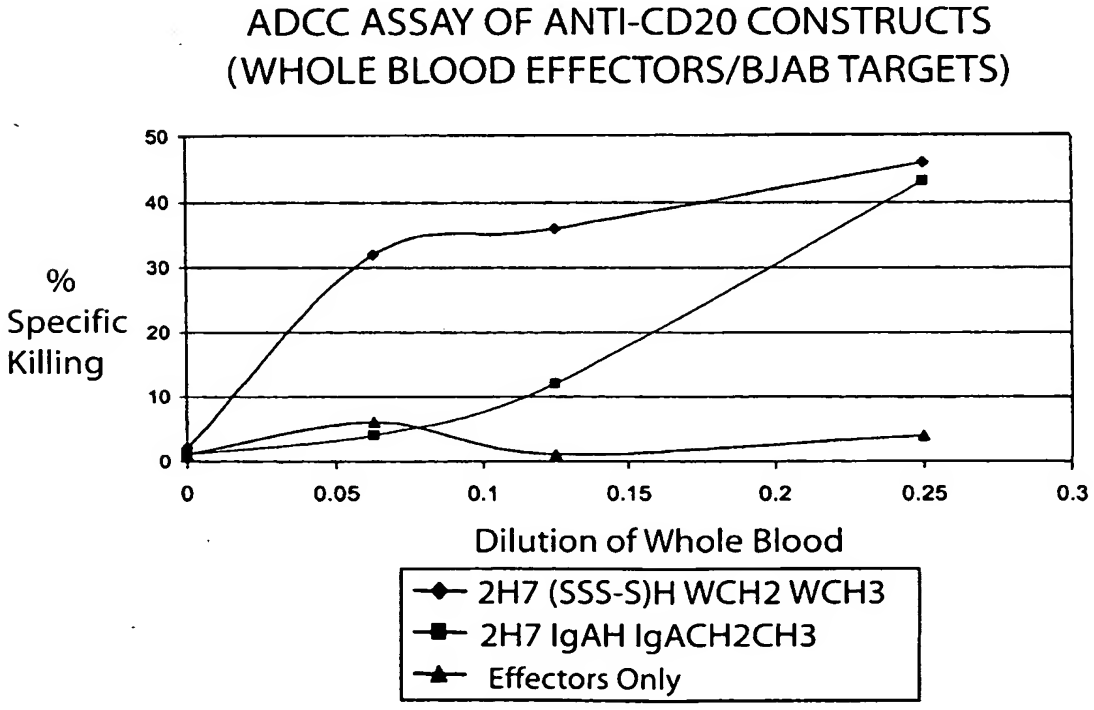


FIG.40

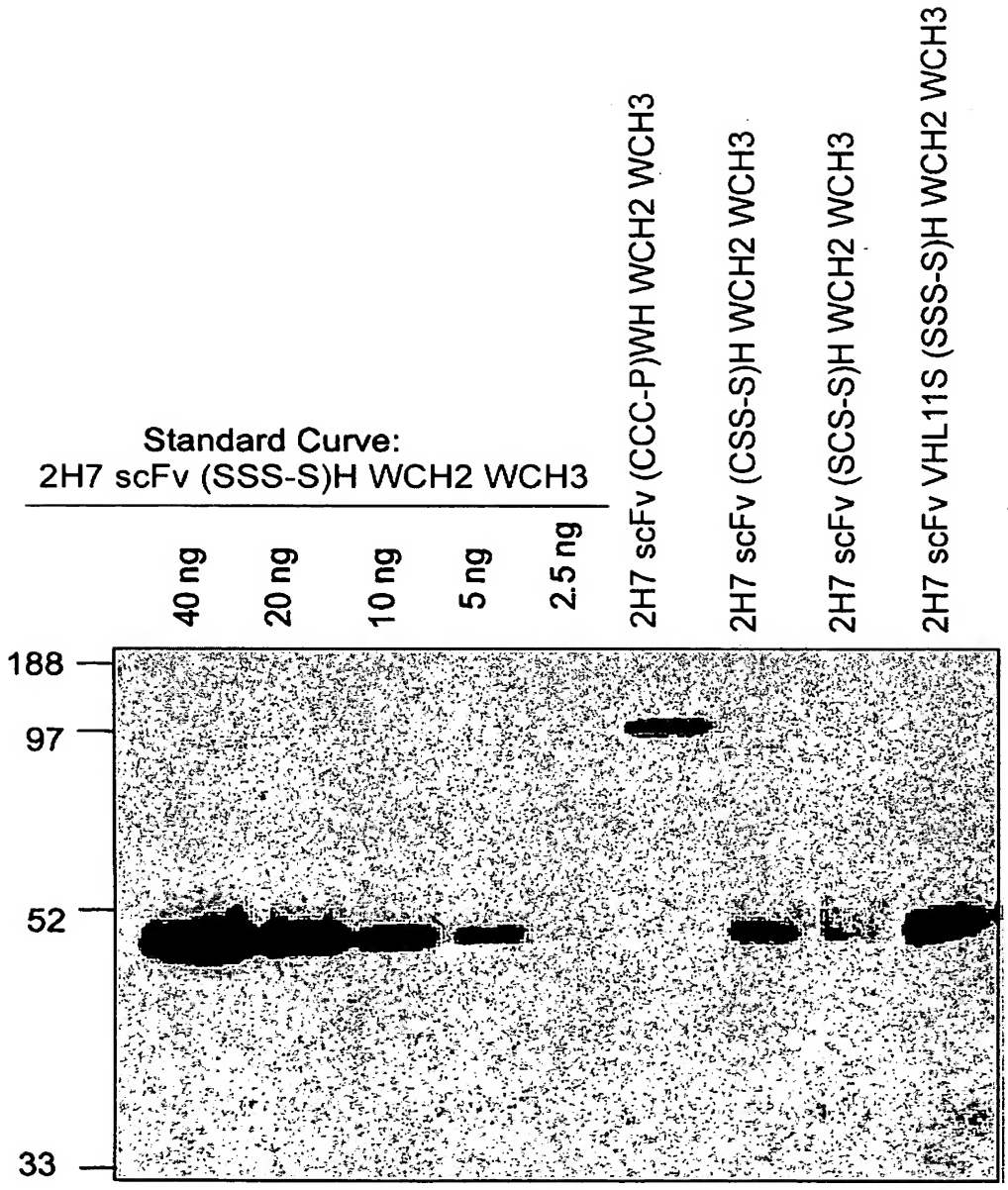

Standard Curve:
2H7 scFv (SSS-S)H WCH2 WCH3
40 ng
20 ng
10 ng
5 ng
2.5 ng
2H7 scFv (CCC-P)WH WCH2 WCH3
2H7 scFv (CSS-S)H WCH2 WCH3
2H7 scFv (SCS-S)H WCH2 WCH3
2H7 scFv VHL11S (SSS-S)H WCH2 WCH3
188
97
52
33

FIG.41A

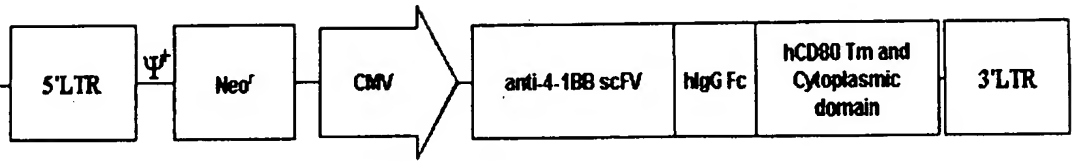



FIG.41B

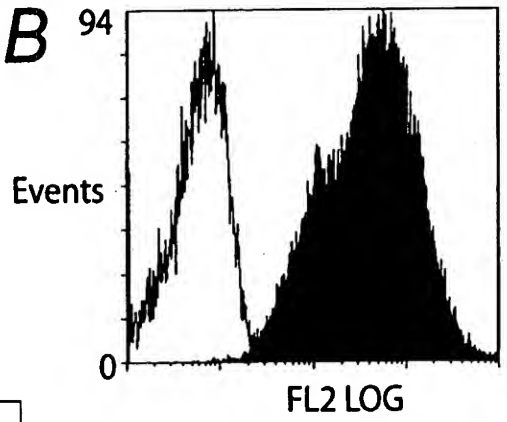



FIG.41C

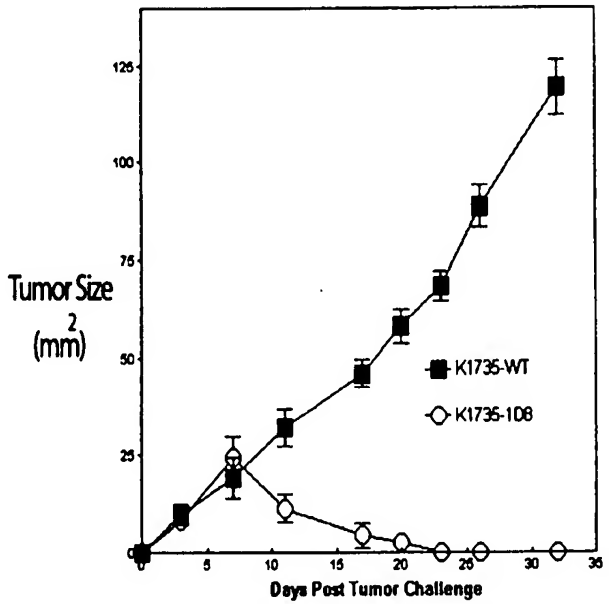



FIG.41D

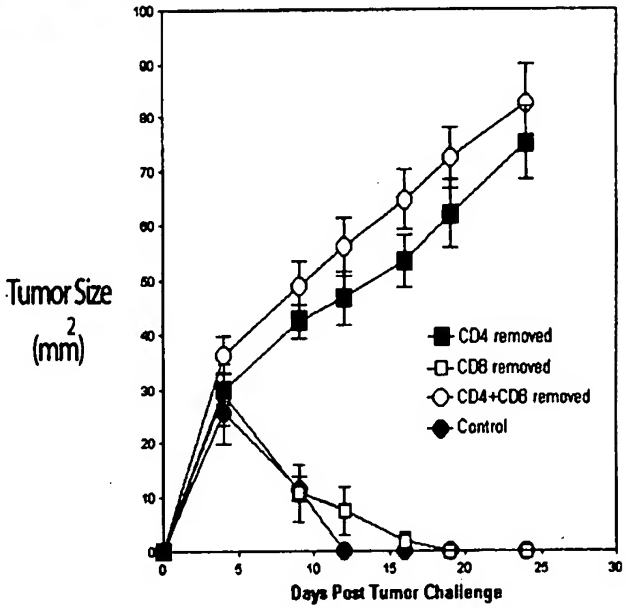


FIG.42

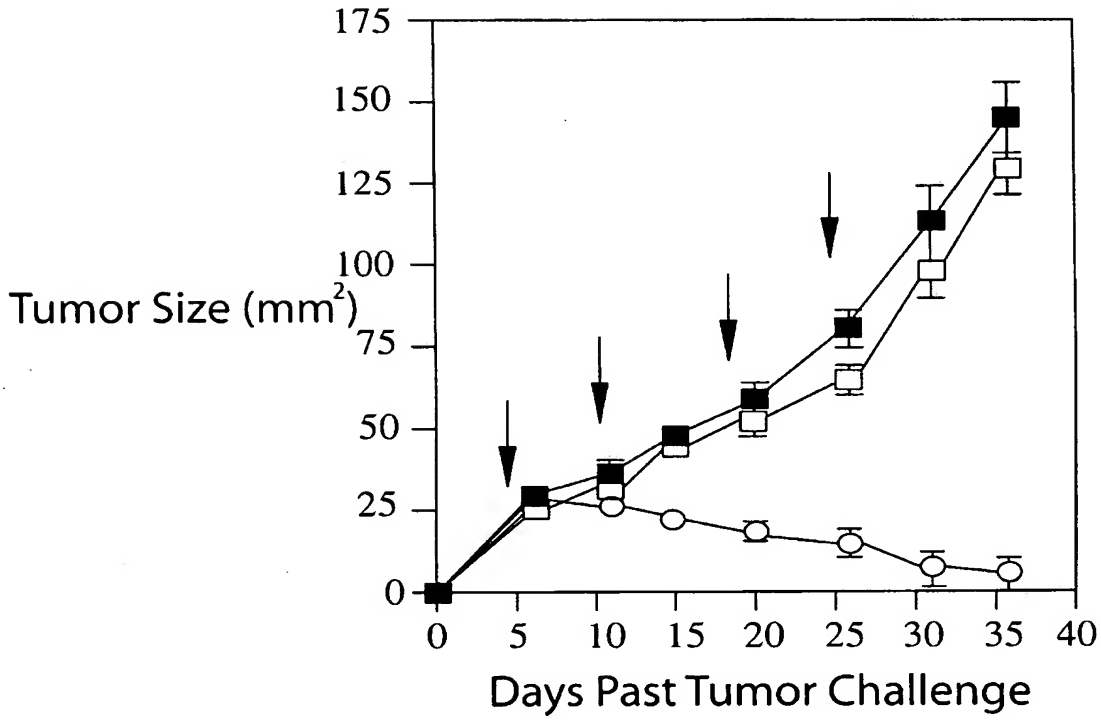

175
150
125
100
75
50
25
0
Tumor Size (mm2)
0
5
10
15
20
25
30
35
40
Days Past Tumor Challenge

FIG.43

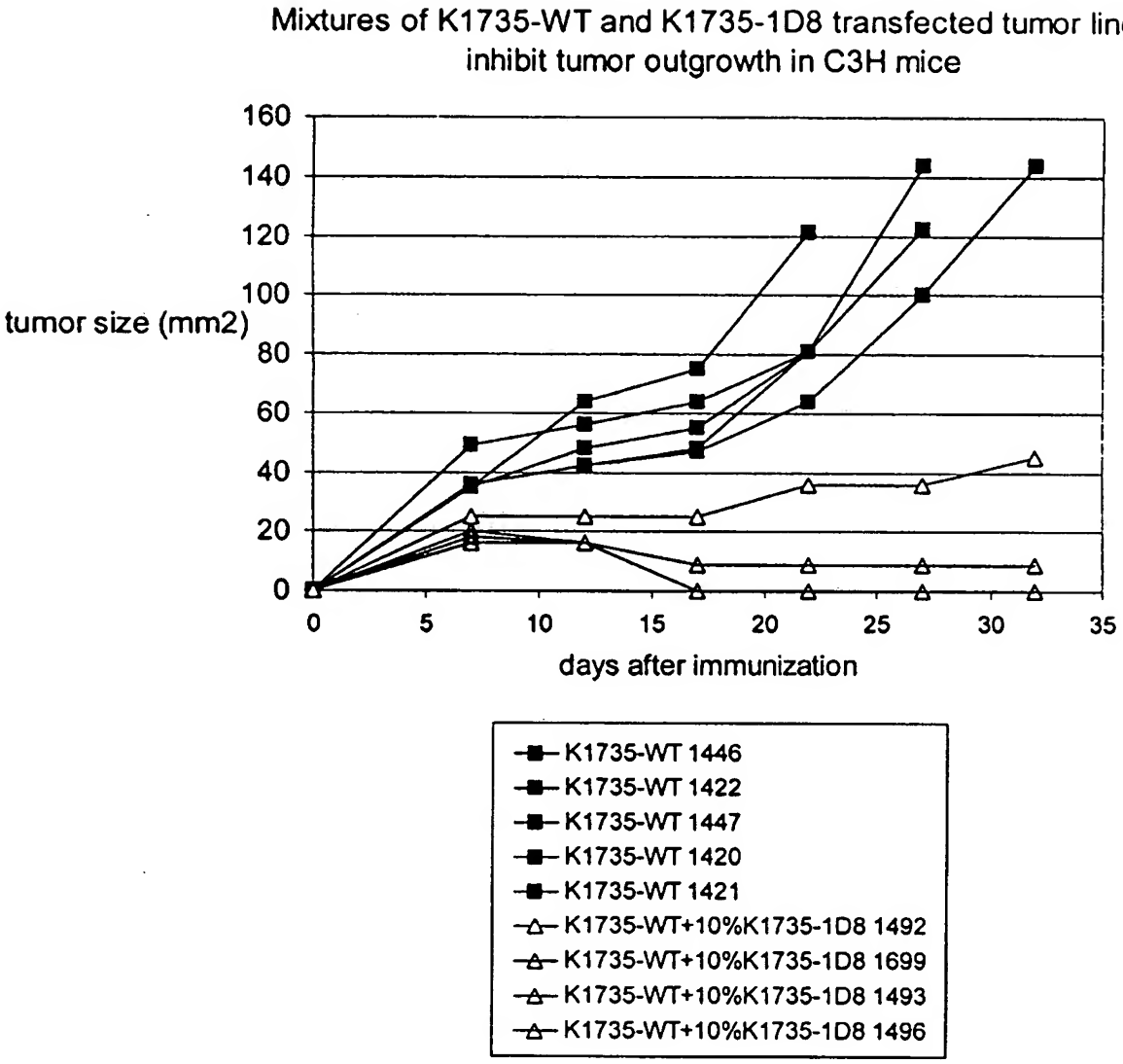

Mixtures of K1735-WT and K1735-1D8 transfected tumor lines inhibit tumor outgrowth in C3H mice
tumor size (mm2)
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
days after immunization
K1735-WT 1446
K1735-WT 1422
K1735-WT 1447
K1735-WT 1420
K1735-WT 1421
K1735-WT+10%K1735-1D8 1492
K1735-WT+10%K1735-1D8 1699
K1735-WT+10%K1735-1D8 1493
K1735-WT+10%K1735-1D8 1496

FIG.44

Expression of 1D8 scFv (SSS-S)H P238SCH2 WCH3 (Anti-CD37) on the Surface of Panned Ag104 Transfected Tumor Cells

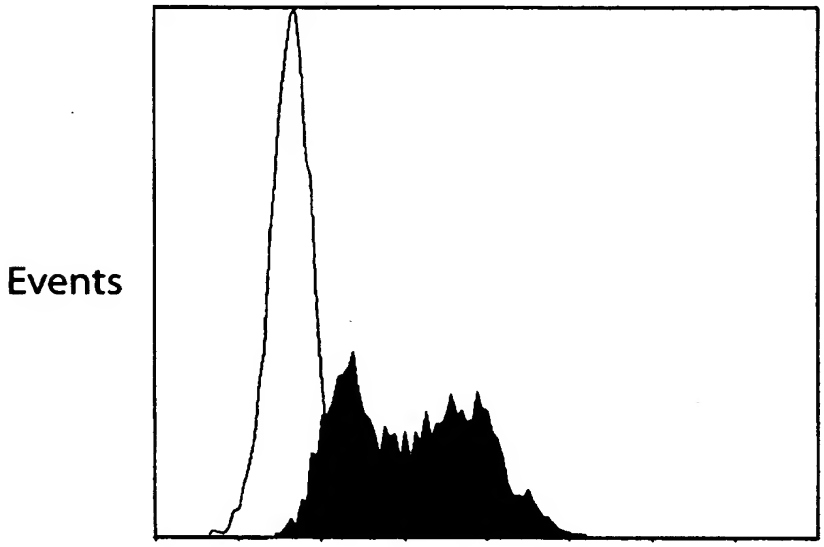


FIG.45

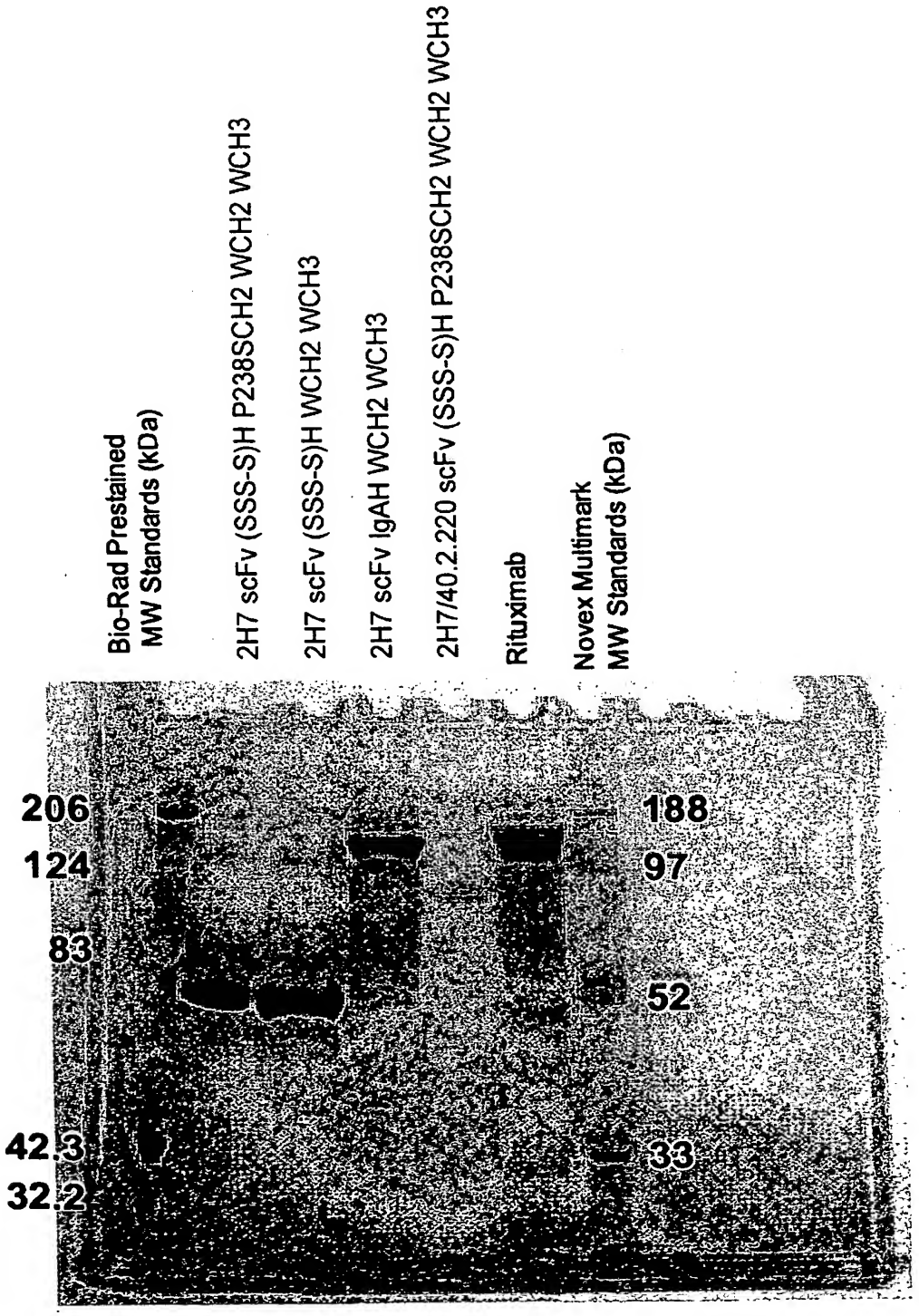

Bio-Rad Prestained
MW Standards (kDa)
2H7 scFv (SSS-S)H P238SCH2 WCH2 WCH3
2H7 scFv (SSS-S)H WCH2 WCH3
2H7 scFv IgAH WCH2 WCH3
2H7/40.2.220 scFv (SSS-S)H P238SCH2 WCH2 WCH3
Rituximab
Novex Multimark
MW Standards (kDa)
206
124
83
42.3
32.2
188
97
52
33

FIG.46

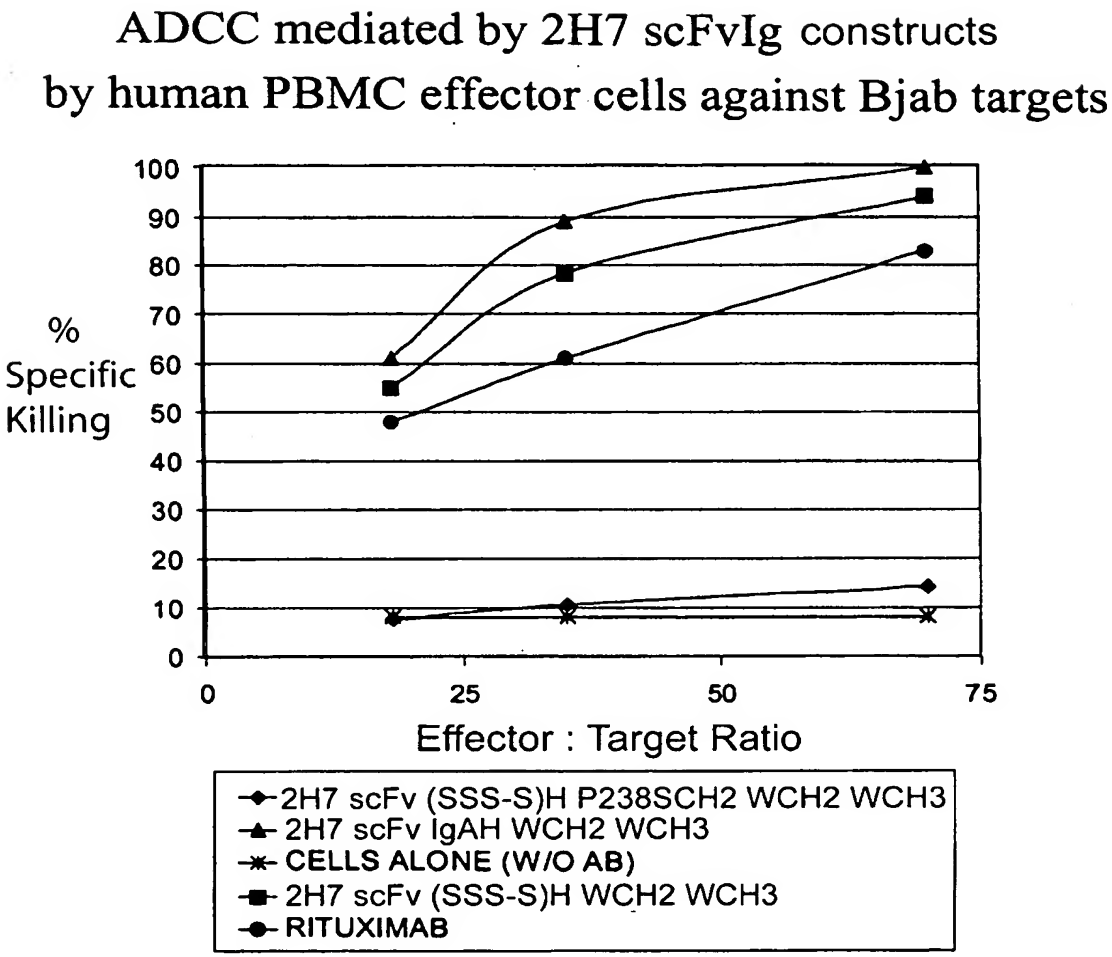

ADCC mediated by 2H7 scFvIg constructs
by human PBMC effector cells against Bjab targets
% Specific Killing
100
90
80
70
60
50
40
30
20
10
0
0
25
50
75
Effector : Target Ratio
2H7 scFv (SSS-S)H P238SCH2 WCH2 WCH3
2H7 scFv IgAH WCH2 WCH3
CELLS ALONE (W/O AB)
2H7 scFv (SSS-S)H WCH2 WCH3
RITUXIMAB

FIG.47

Cell surface expression of
G19-4scFv (SSS-S)H P238SCH2 WCH3
fusion protein on Reh and T51 Cells.

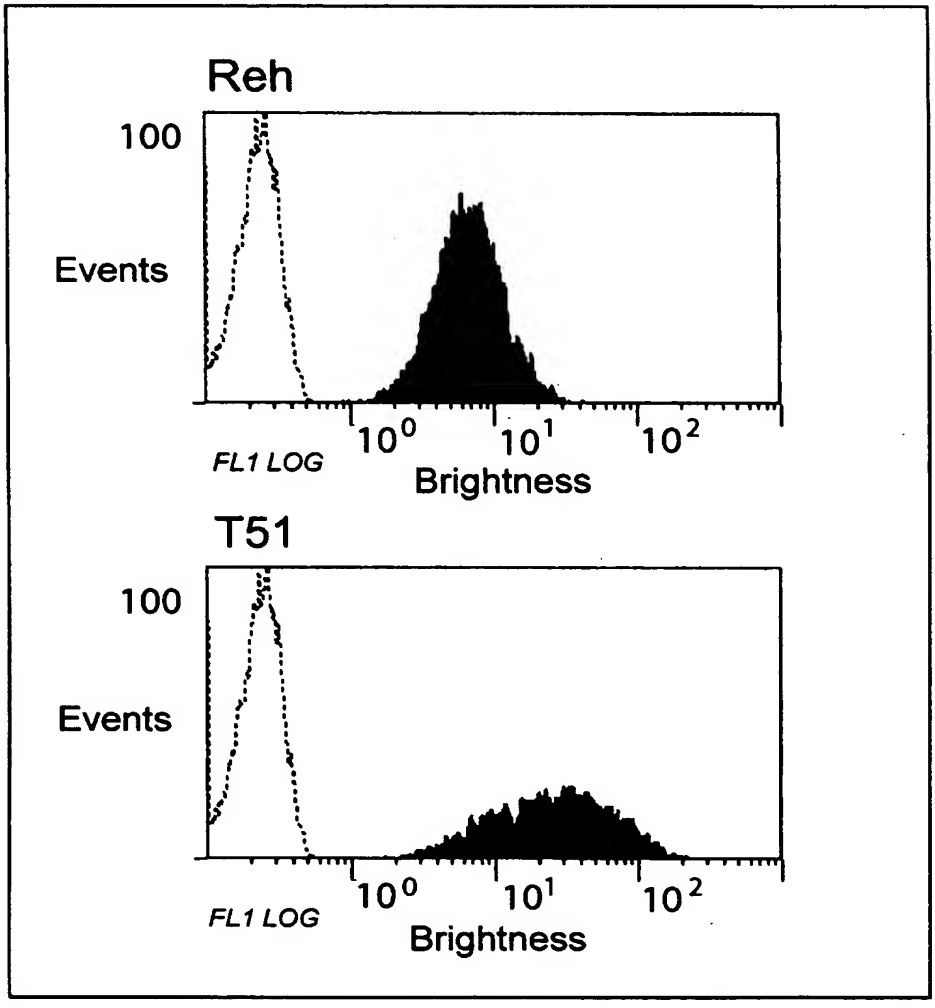


FIG.48

Targeting of Cytotoxicity to Transfected Cell Lines
by Surface expression of
G19-4 scFv (SSS-S)H P238SCH2 WCH3

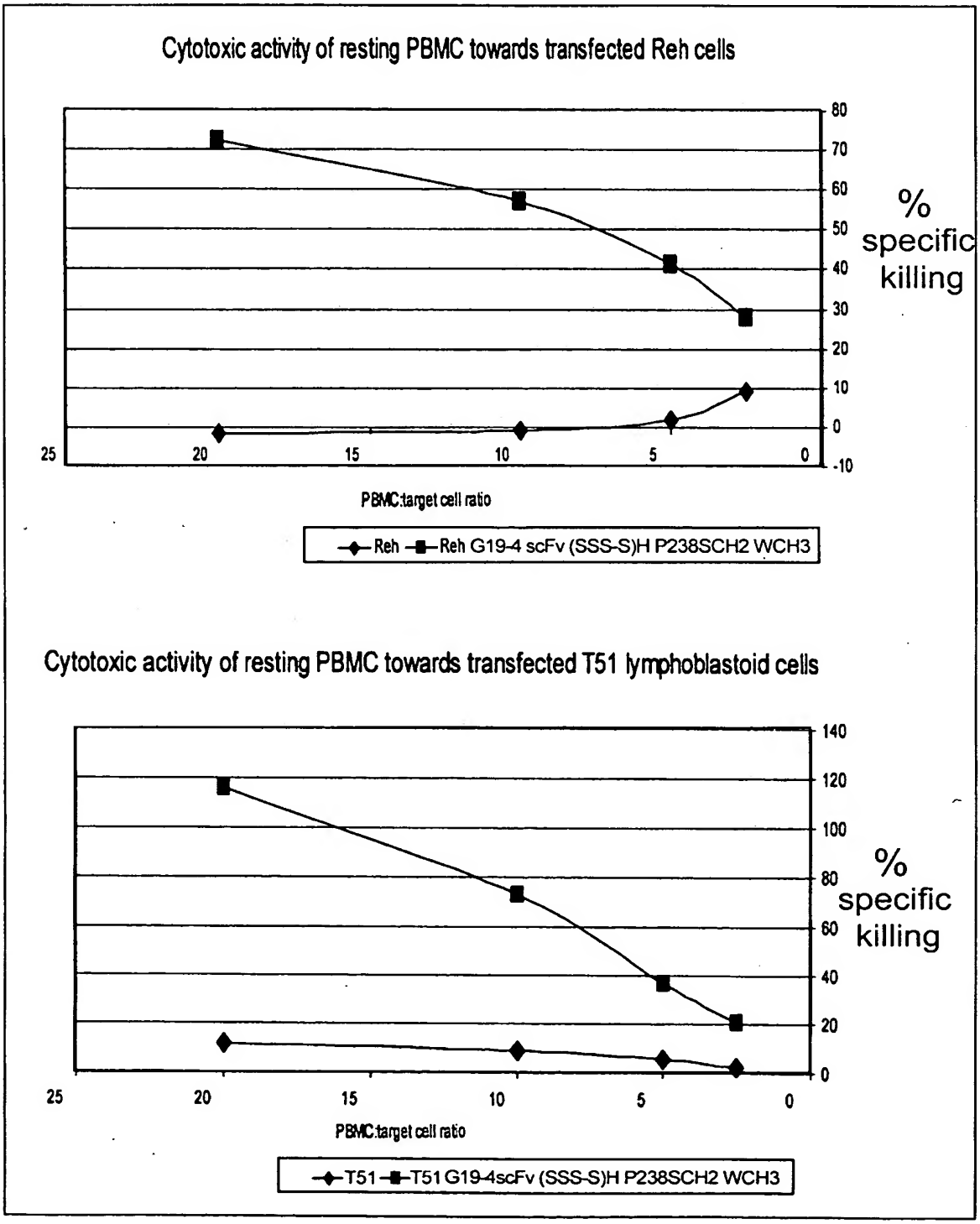
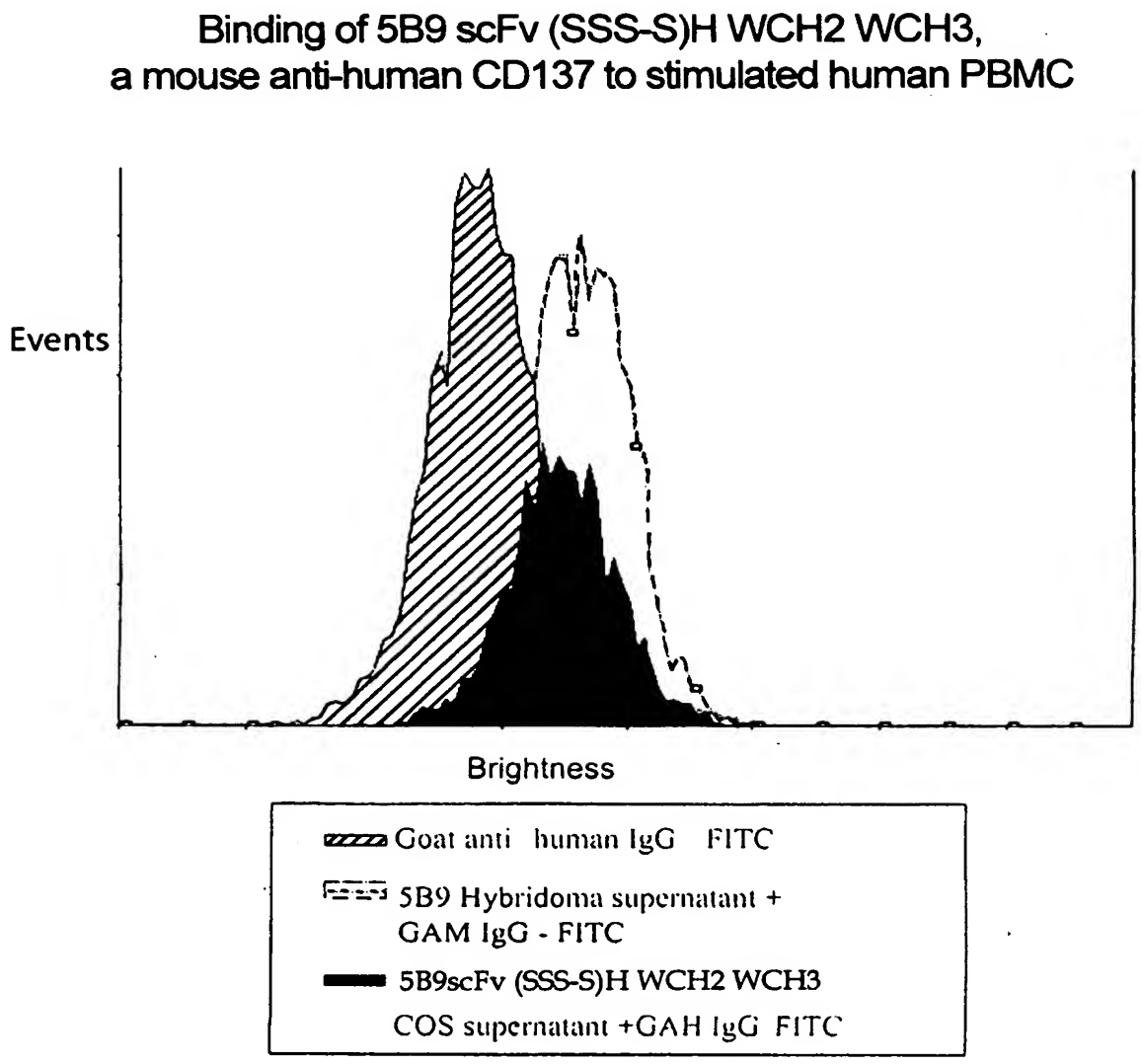
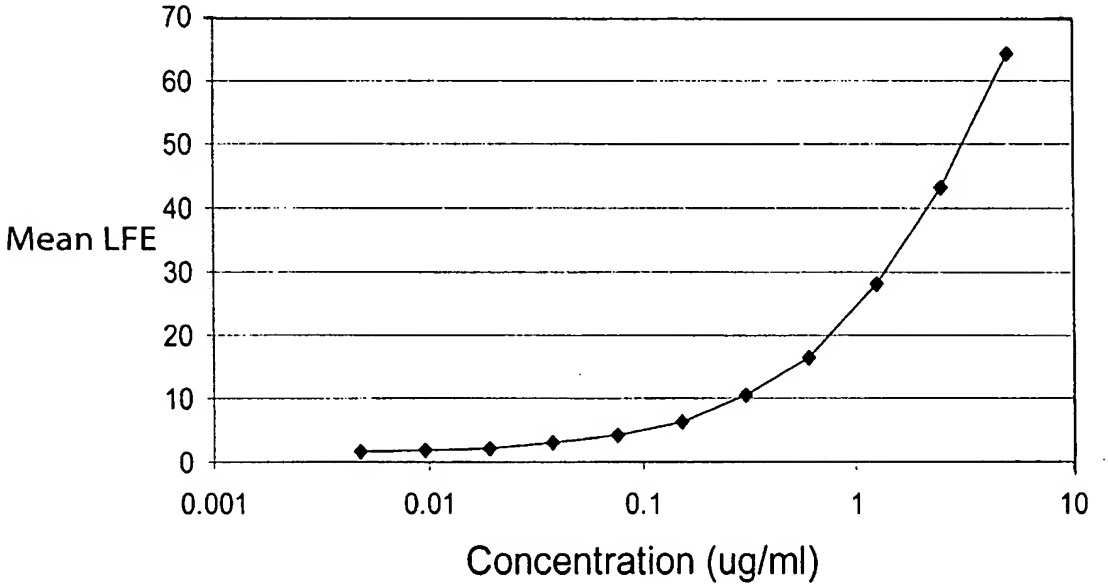


FIG.49


Binding of 5B9 scFv (SSS-S)H WCH2 WCH3,
a mouse anti-human CD137 to stimulated human PBMC
Events
Brightness
Goat anti human IgG FITC
5B9 Hybridoma supernatant +
GAM IgG - FITC
5B9scFv (SSS-S)H WCH2 WCH3
COS supernatant +GAH IgG FITC

# FIG.50A

Effect of $V_H$L11S Mution on
2H7 scFv (SSS-S)H WCH2 WCH3 Protein Expression




# FIG.50B

Effect of $V_H$L11S Mutation on
2H7 scFv (SSS-S)H WCH2 WCH3 Protein Expression

CHO supernatant Brightness and Estimation of Protein concentrations from Standard Curve:

| | CHO clone name | | | | |
|---|---|---|---|---|---|
| | **4F2** | **4F5** | **3E5** | **6B11A** | **2B8A** |
| Mean LFE | | | | | |
| 1/100 | 71.7 | 40.6 | 31.5 | 99.7 | 101.5 |
| 1/500 | 27.1 | 12.4 | 11.2 | 40.8 | 43 |
| **approx conc. µg/ml** | **600** | **225** | **125** | **1000** | **1250** |

FIG.51

Production Levels of 2H7scFv VH L11S
(SSS-S)H WCH2 WCH3
From CHO Clone Culture Supernatants

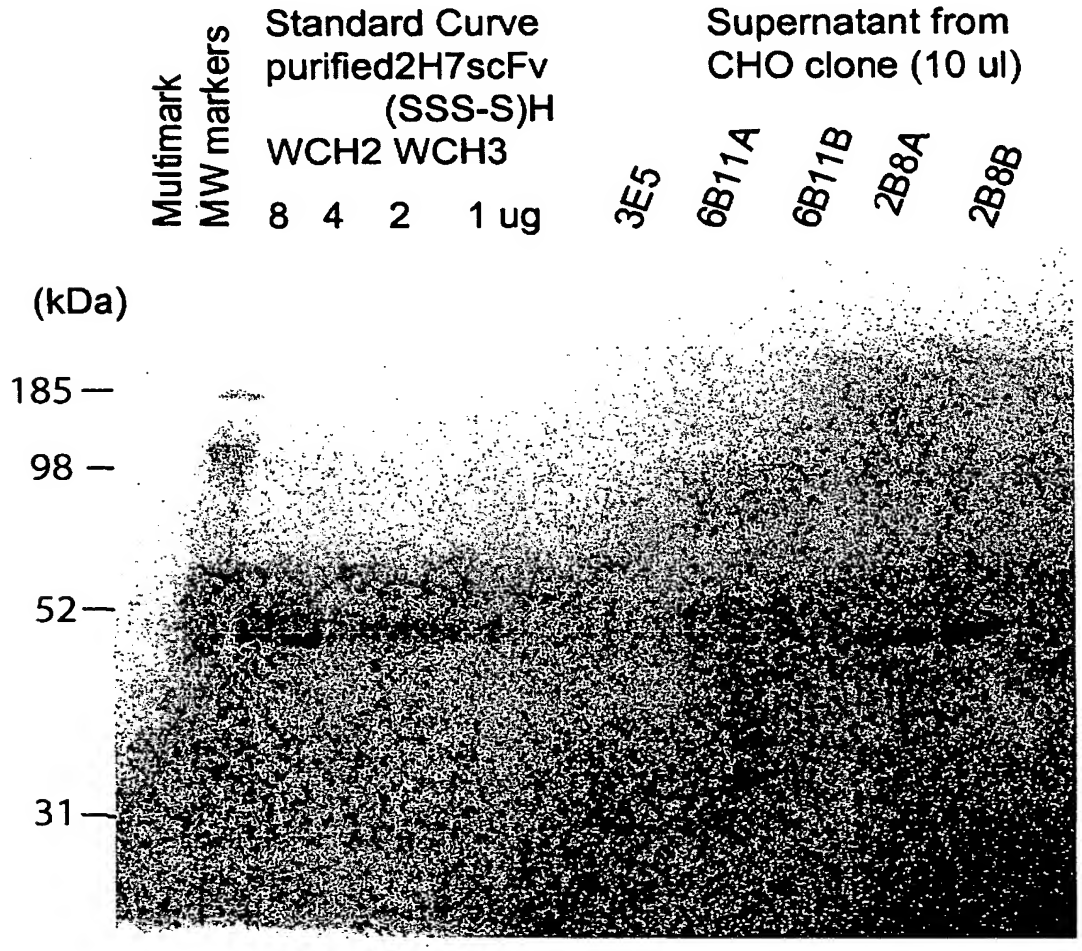
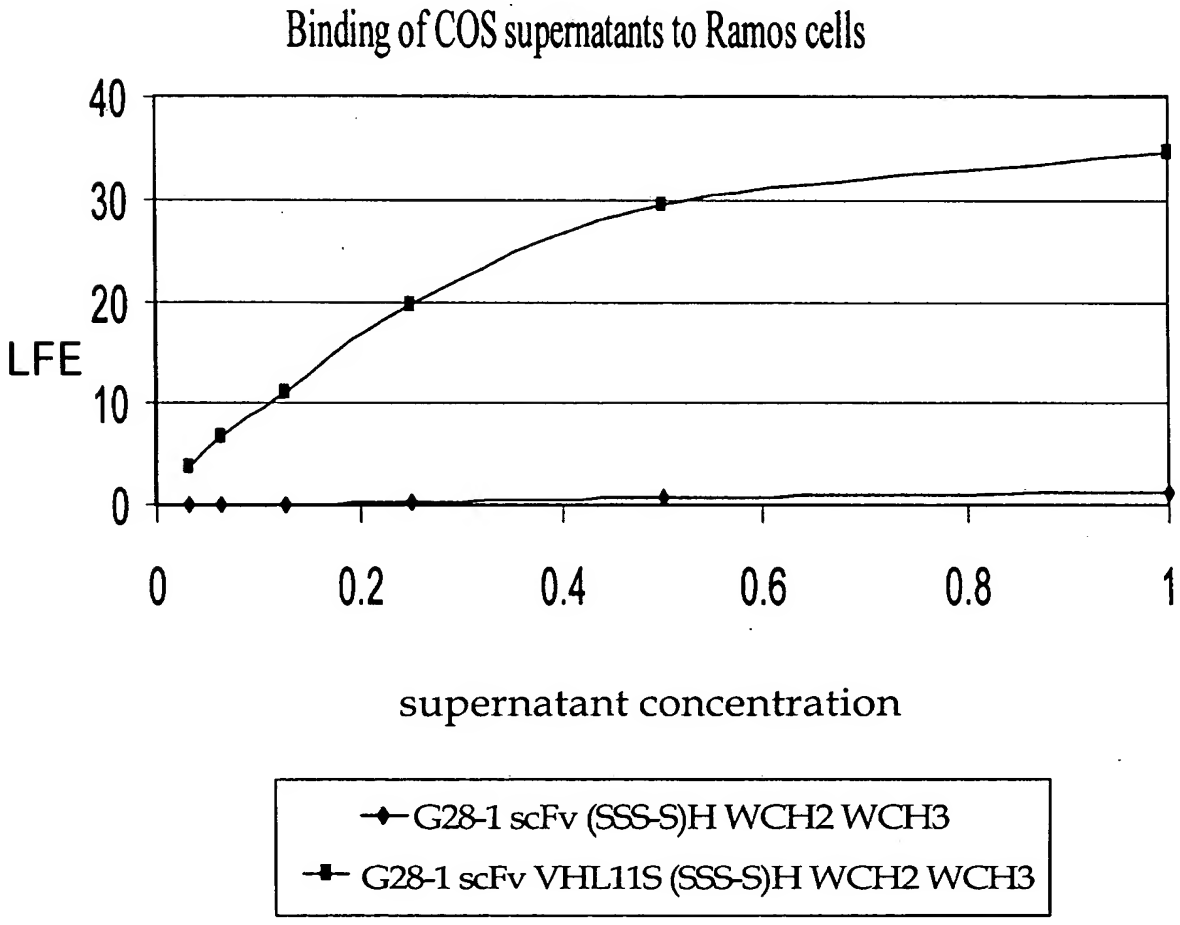


FIG.52

Effect of VHL11S Mutation on G28-1 scFvIg Construct Protein Production from COS cells



# FIG.53A

## Immunoblot of G28-1 scFvIg Constructs

Increased Protein Levels in COS supernatants
transfected with G28-1scFv (SSS-S)H WCH2 WCH3
After Substitution of Leucine with Serine at position 11 of VH (VHL11S)


# FIG.53B

## Immunoblot of G28-1 scFvIg Constructs

Increased Protein Levels in COS supernatants
transfected with G28-1scFv (SSS-S)H WCH2 WCH3
After Substitution of Leucine with Serine at position 11 of VH (VHL11S)

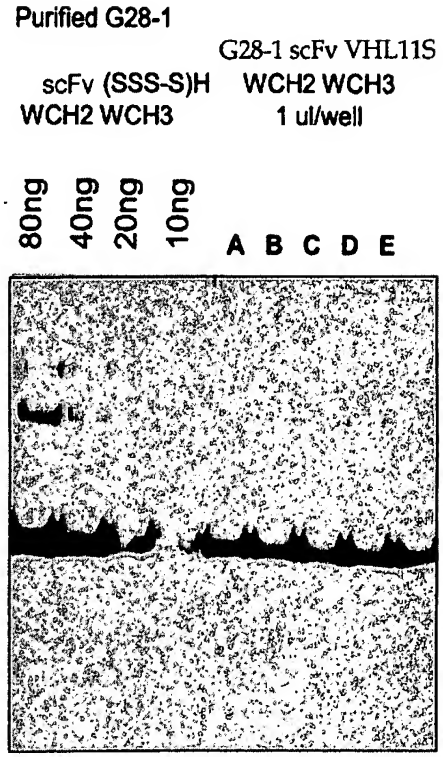


FIG.54

Binding of 2H7 scFvIg Constructs with Altered Hinges and CH3 domains to CD20 CHO Cells

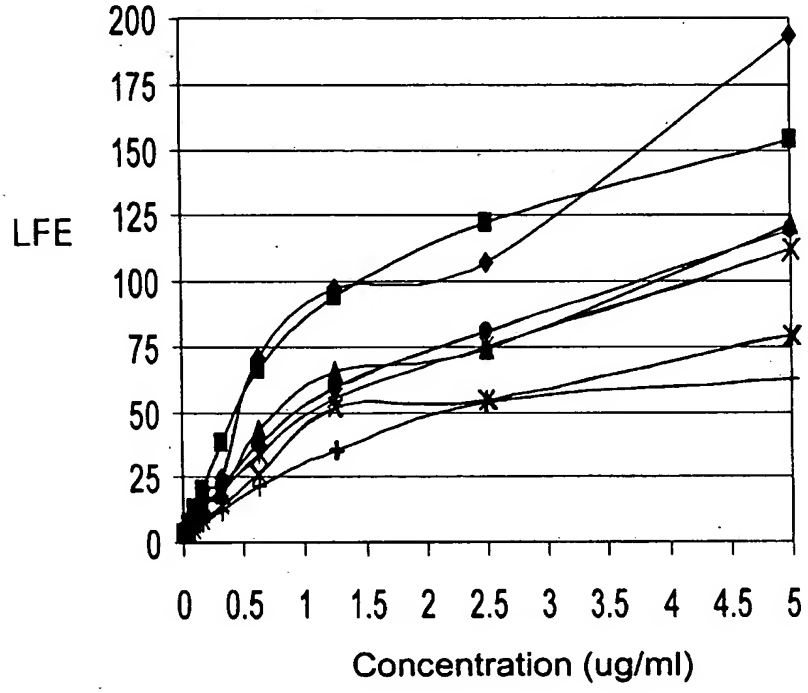


FIG.55

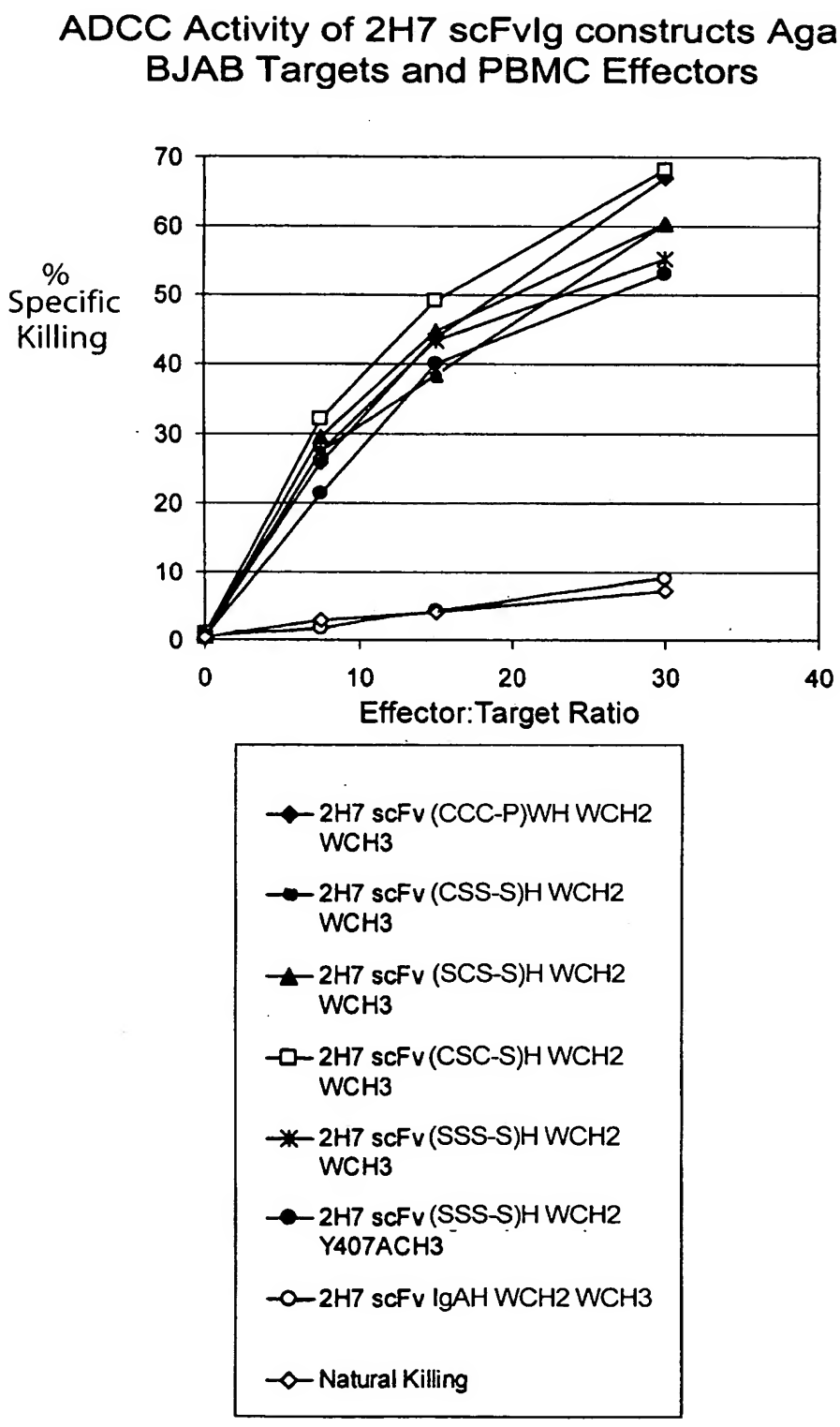

ADCC Activity of 2H7 scFvIg constructs Against BJAB Targets and PBMC Effectors
% Specific Killing
70
60
50
40
30
20
10
0
0
10
20
30
40
Effector:Target Ratio
2H7 scFv (CCC-P)WH WCH2 WCH3
2H7 scFv (CSS-S)H WCH2 WCH3
2H7 scFv (SCS-S)H WCH2 WCH3
2H7 scFv (CSC-S)H WCH2 WCH3
2H7 scFv (SSS-S)H WCH2 WCH3
2H7 scFv (SSS-S)H WCH2 Y407ACH3
2H7 scFv IgAH WCH2 WCH3
Natural Killing

FIG.56

## Complement Activity of 2H7 scFvIg Constructs With Ramos Target Cells

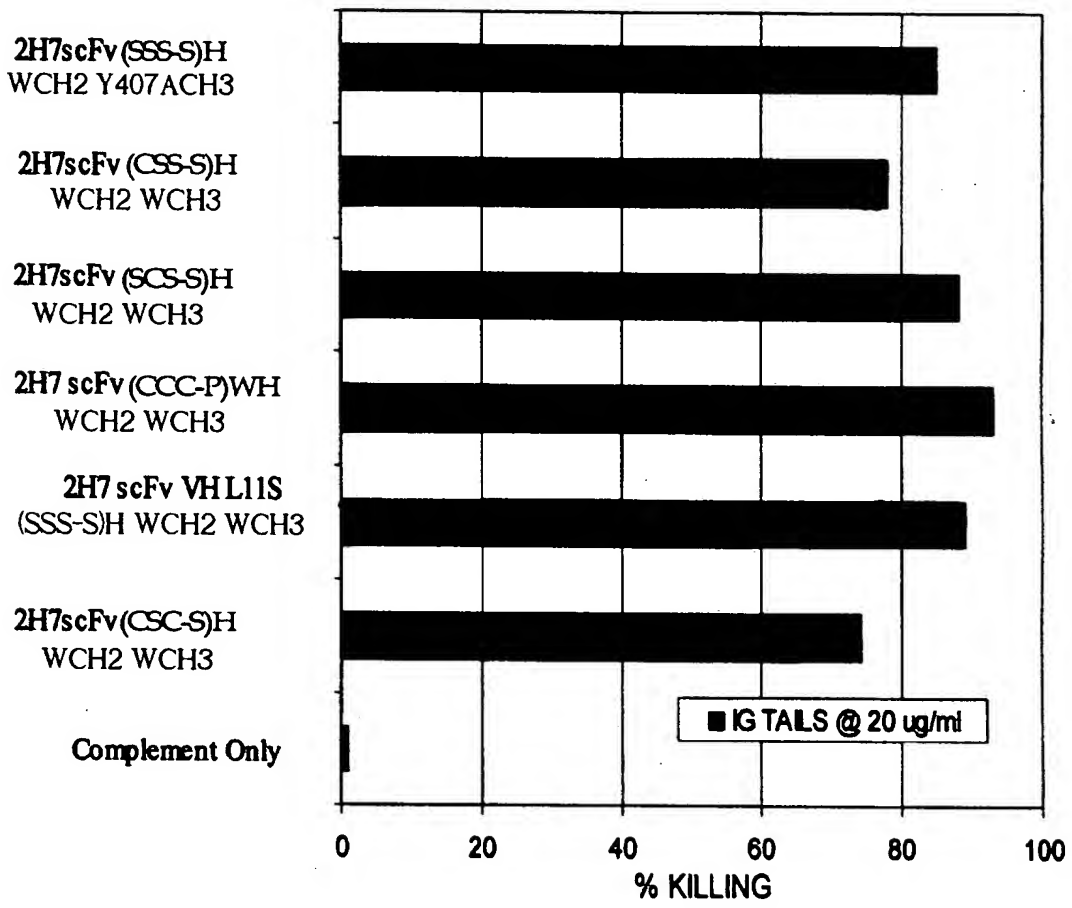
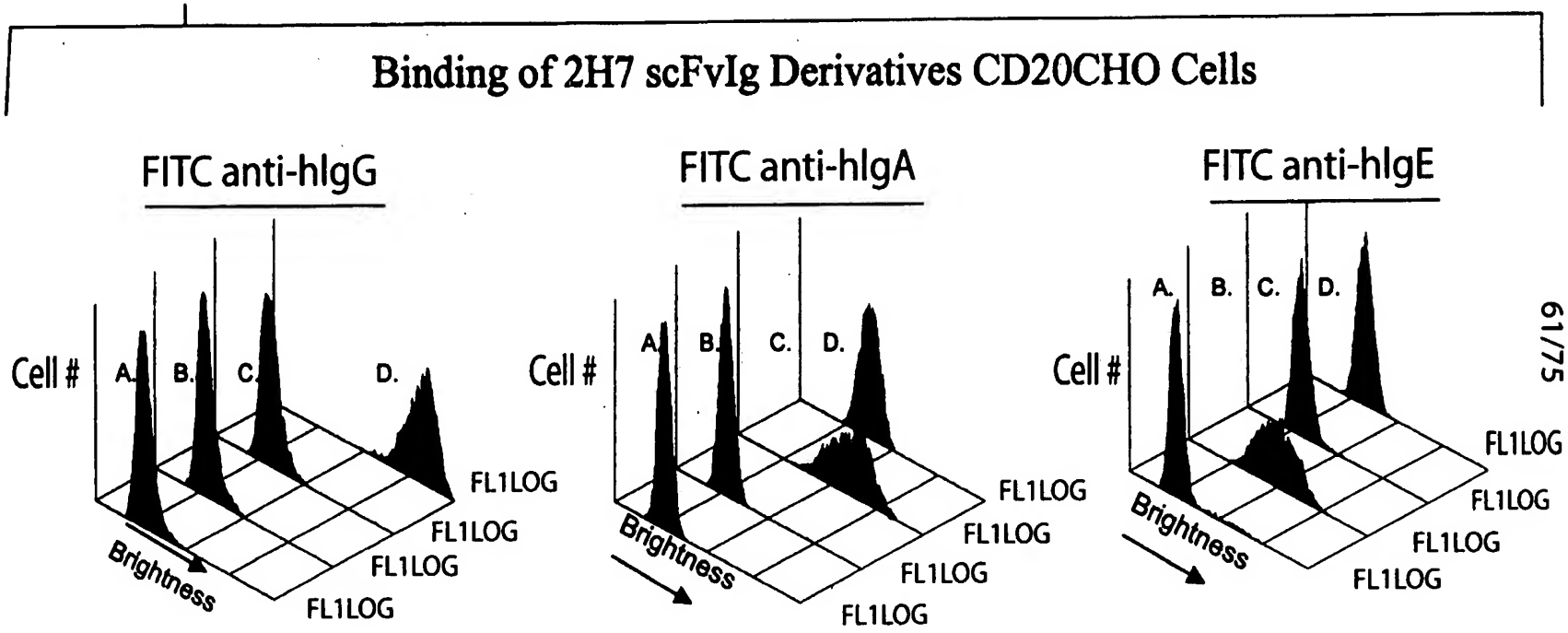


FIG.57




A. No fusion protein

B. 2H7 scFv IgE CH2CH3CH4

C. 2H7 scFv IgAH WCH2 WCH3

D. 2H7 scFv(SSS-S)H WCH2 WCH3

FIG.58A

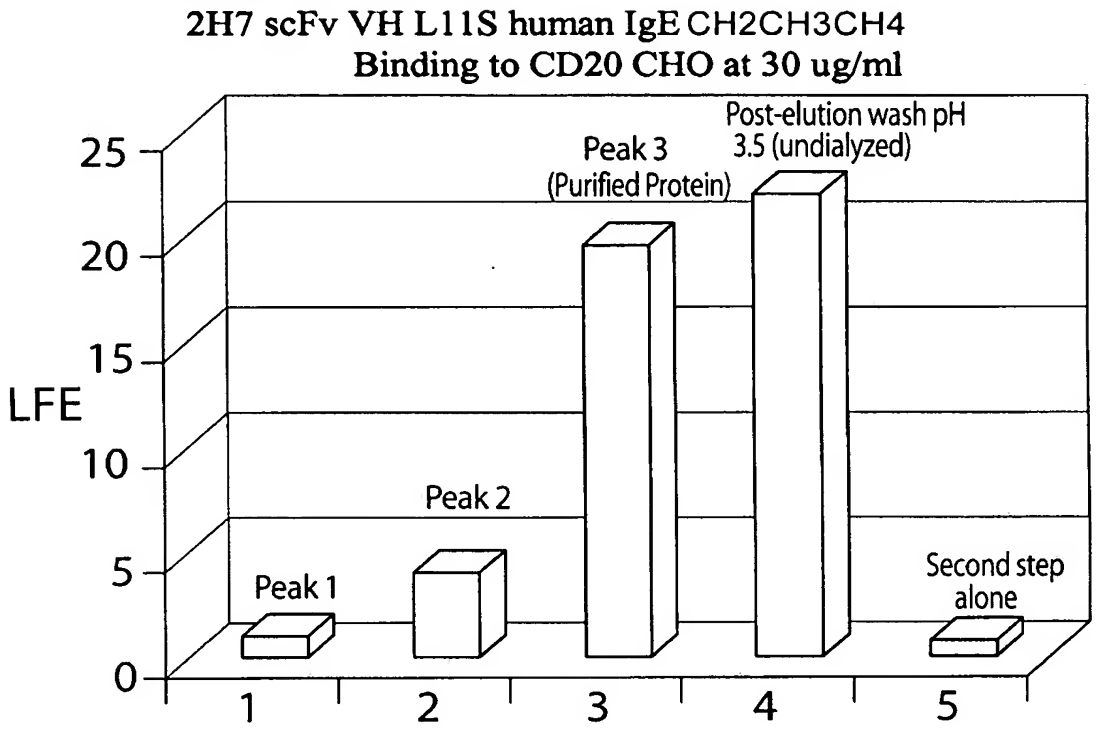



FIG.58B

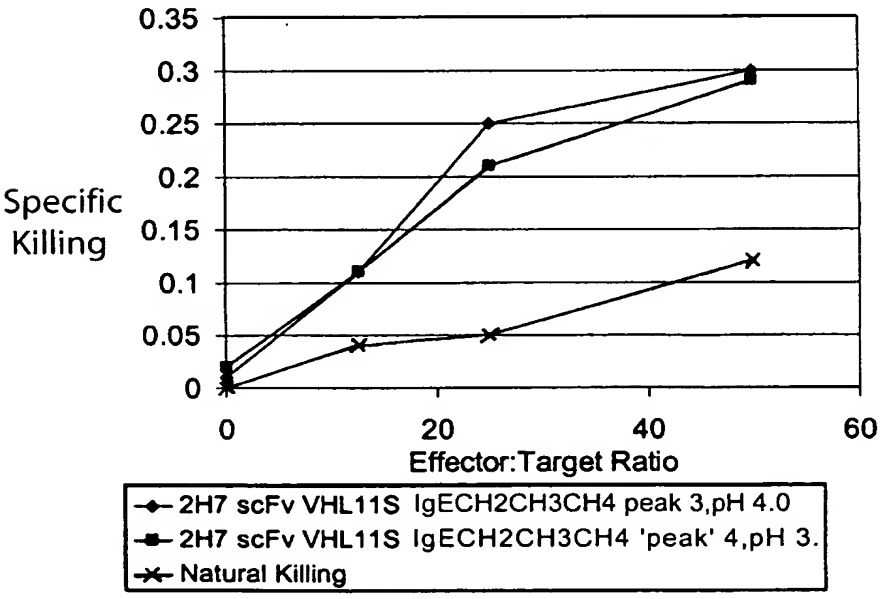


FIG.59

Binding Data For COS derived 2H7 scFv VHL11S mIgECH2CH3CH4 and mIgAH WCH2 WCH3

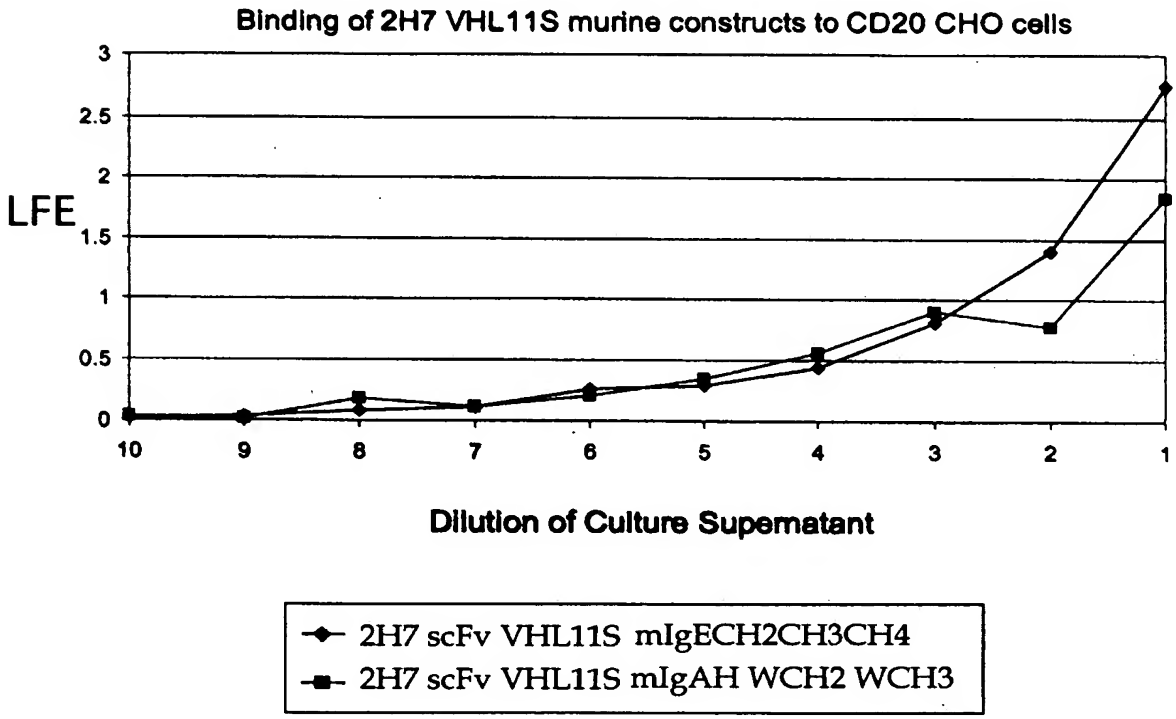


FIG.60A

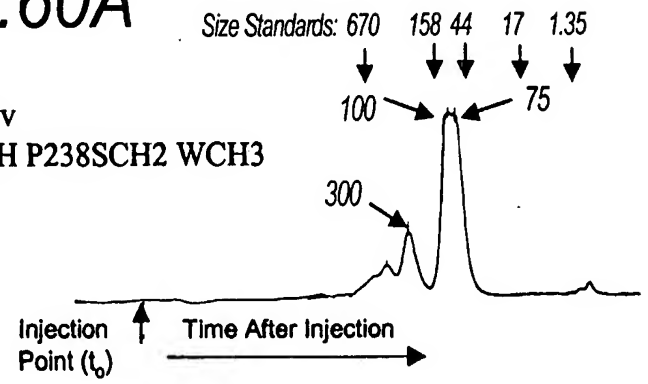



FIG.60B

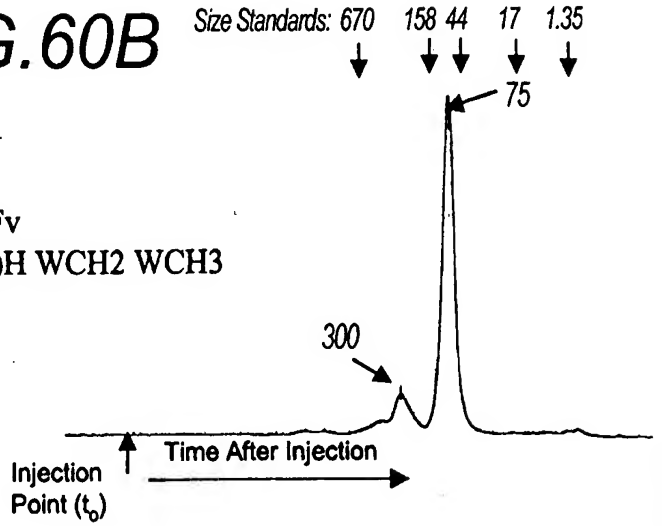



FIG.60C

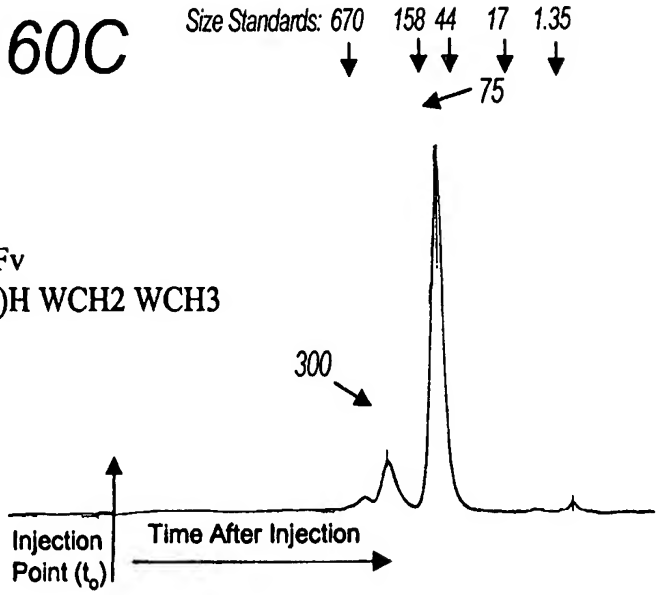



FIG.60D

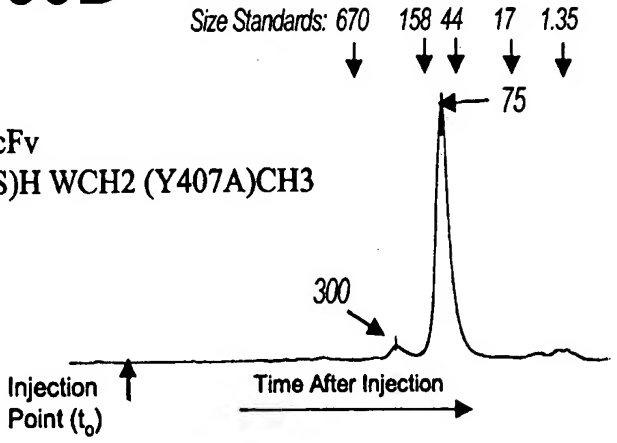


FIG.61A

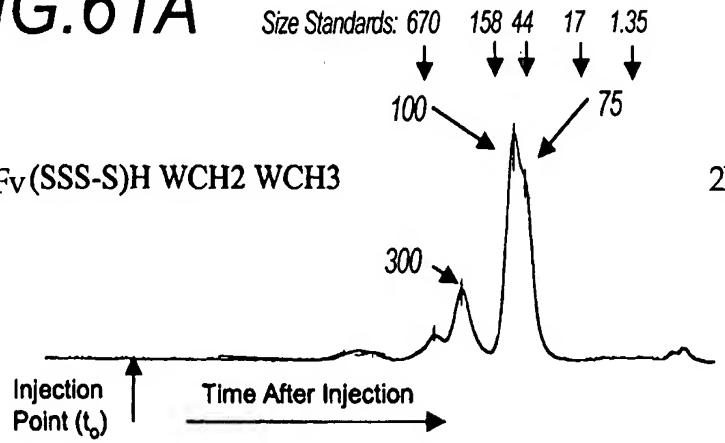



FIG.61B

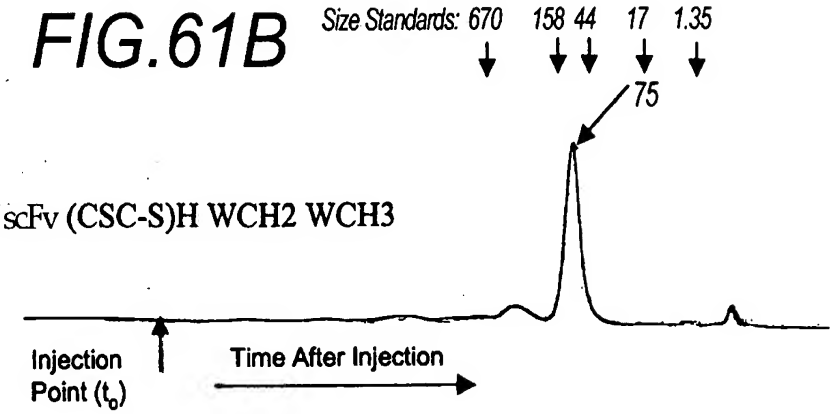





FIG.61C

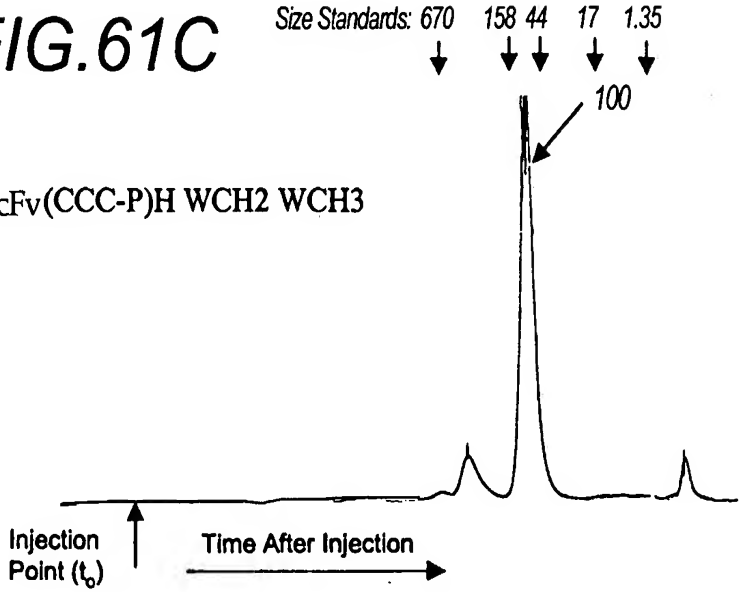



FIG.61D

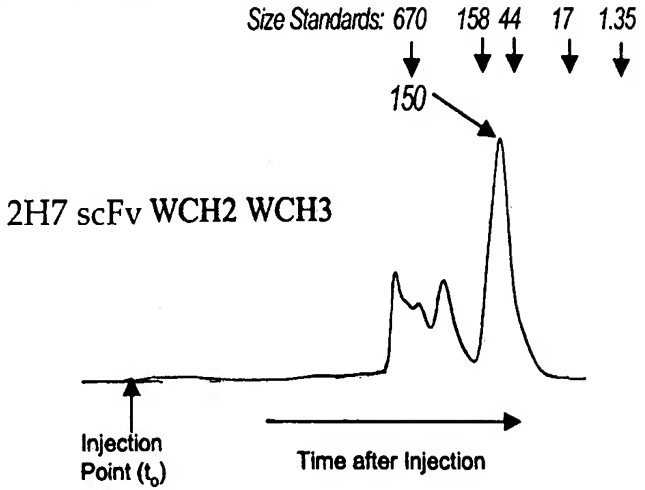


FIG.62A

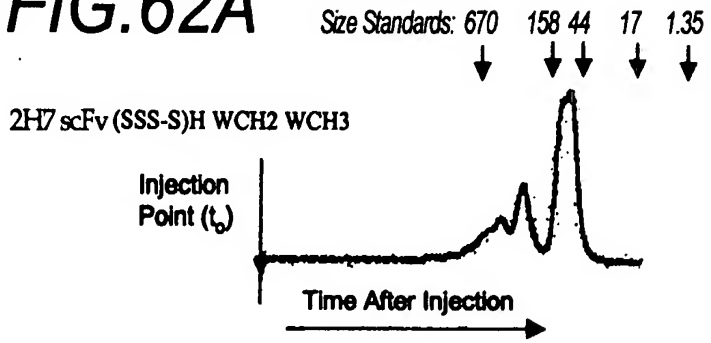



FIG.62B

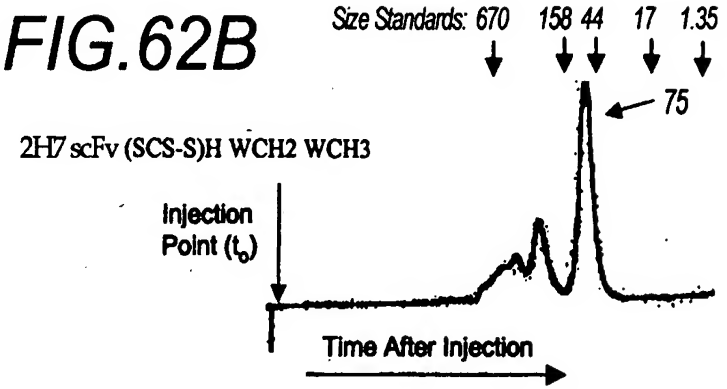





FIG.62C

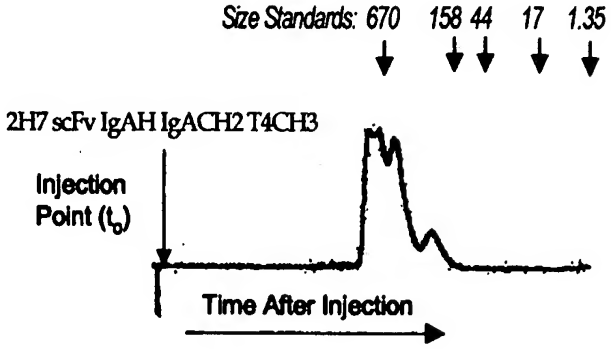



FIG.62D

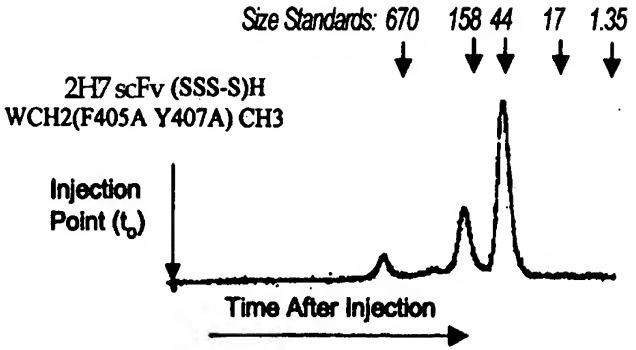


FIG.63

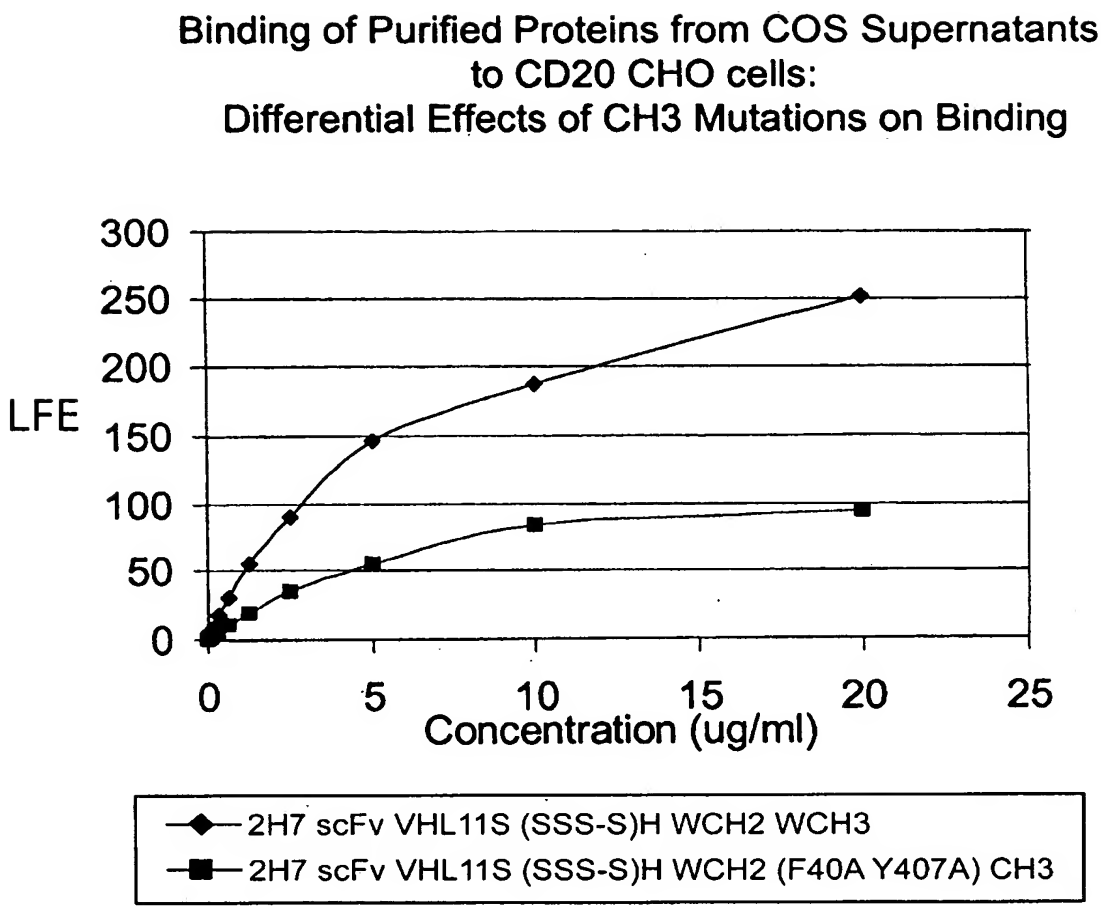

Binding of Purified Proteins from COS Supernatants
to CD20 CHO cells:
Differential Effects of CH3 Mutations on Binding
LFE
300
250
200
150
100
50
0
0
5
10
15
20
25
Concentration (ug/ml)
2H7 scFv VHL11S (SSS-S)H WCH2 WCH3
2H7 scFv VHL11S (SSS-S)H WCH2 (F40A Y407A) CH3

FIG.64

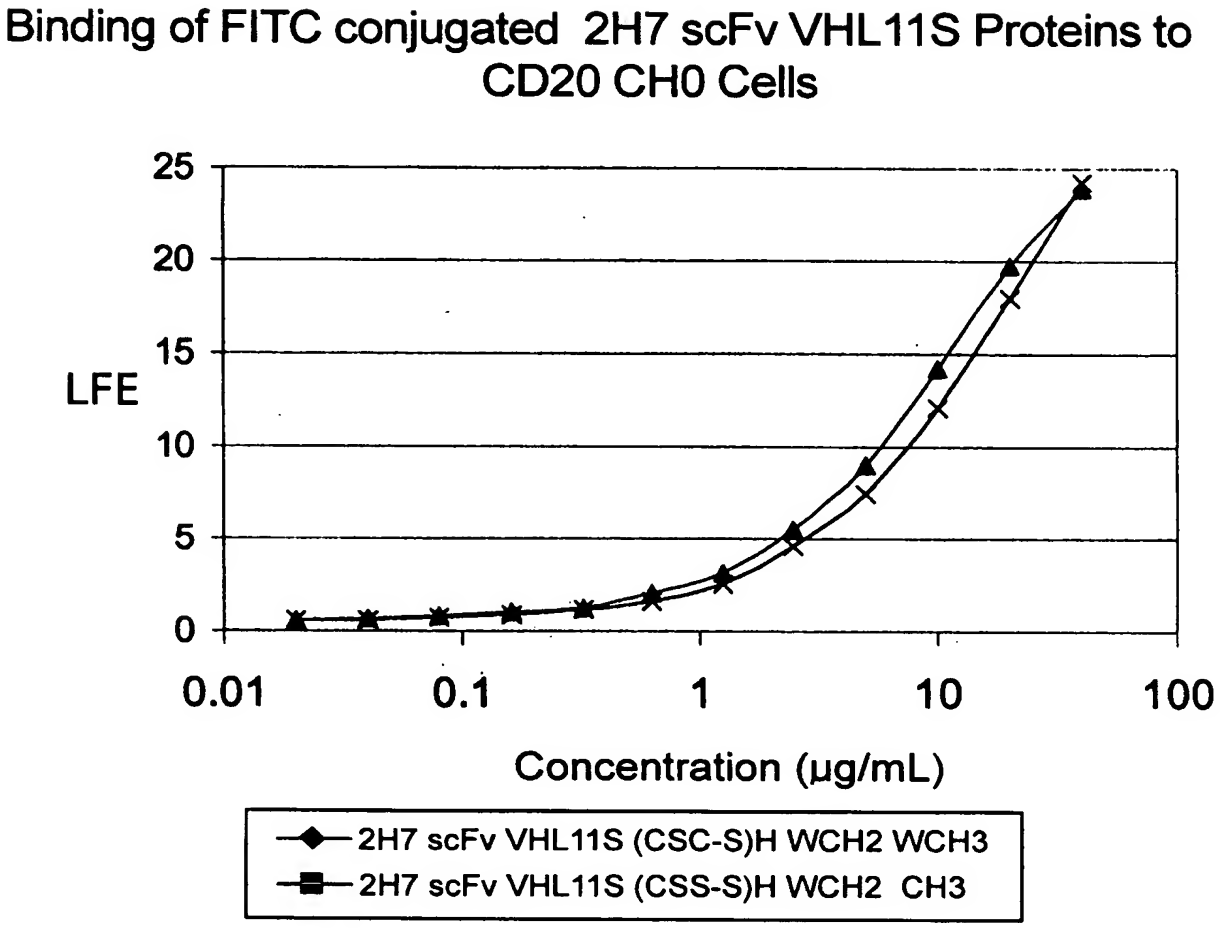

Binding of FITC conjugated 2H7 scFv VHL11S Proteins to CD20 CH0 Cells
LFE
25
20
15
10
5
0
0.01
0.1
1
10
100
Concentration (µg/mL)
2H7 scFv VHL11S (CSC-S)H WCH2 WCH3
2H7 scFv VHL11S (CSS-S)H WCH2 CH3

FIG.65

Nonreducing SDS-PAGE on Protein A-Purified Lots of 2H7 scFv VHL11S Constructs (10 ug/lane)

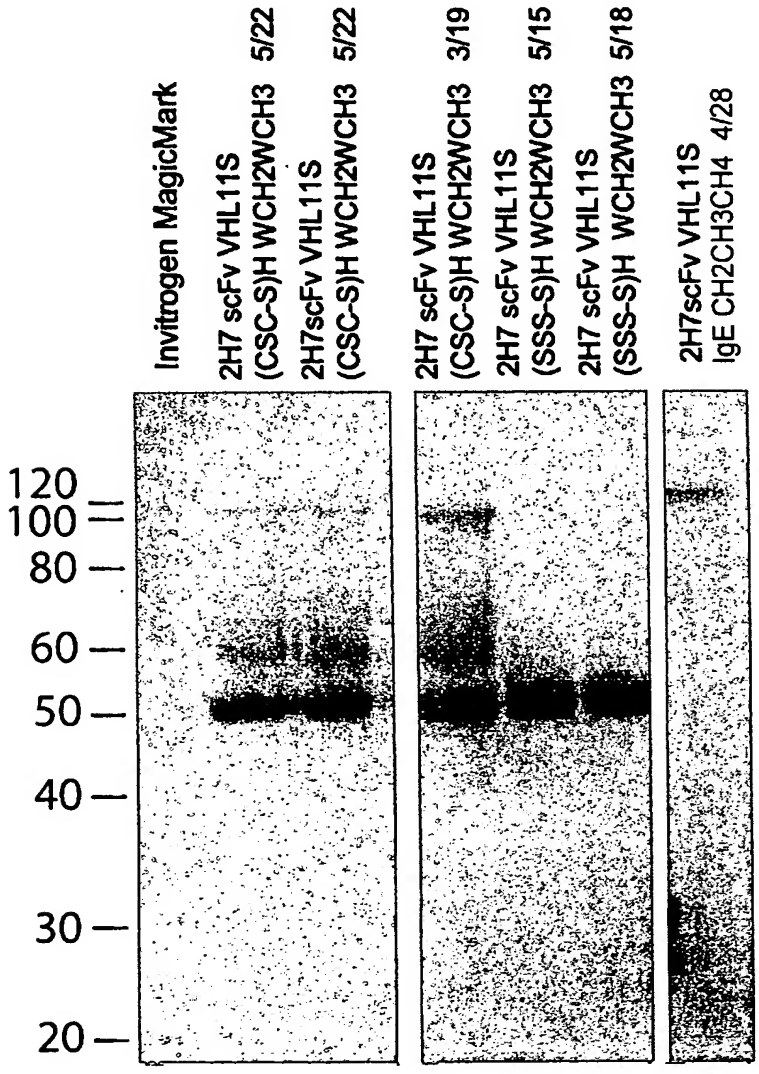
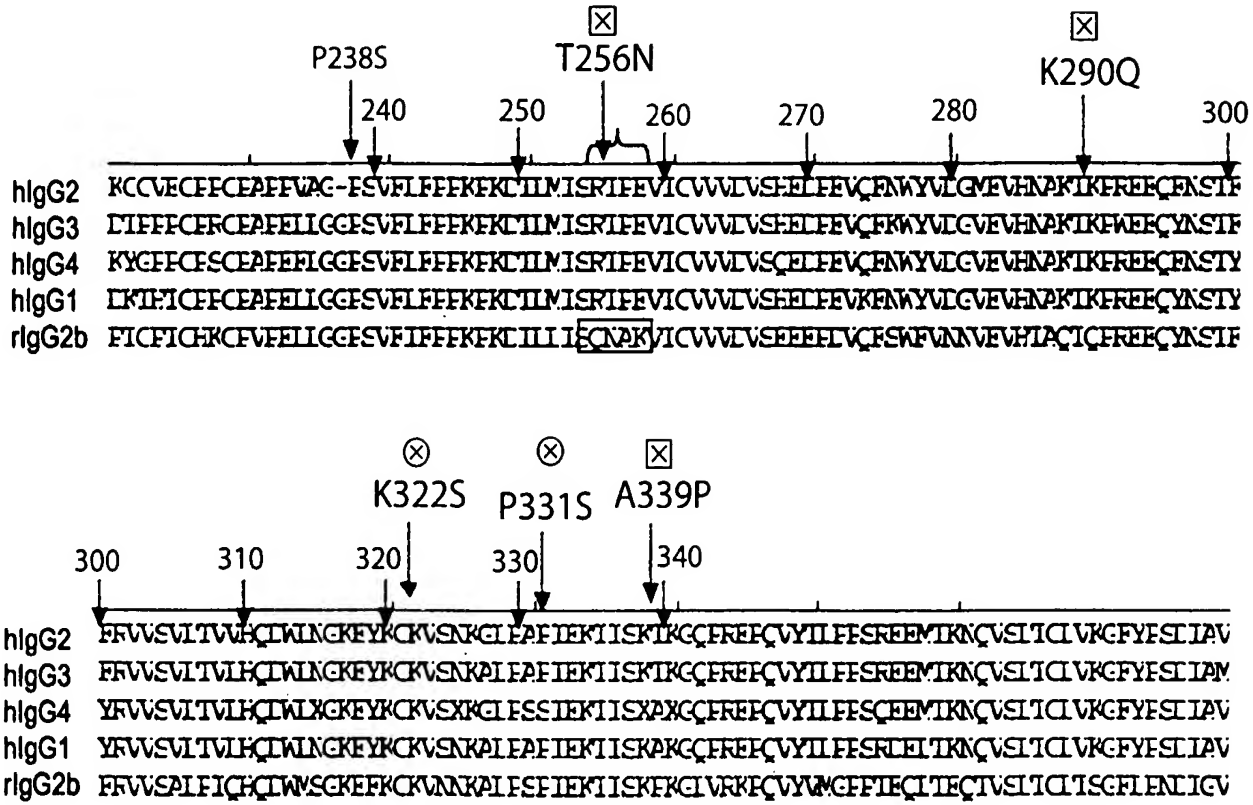


FIG.66

Alterations in Human IgG Fc sequence
that differentially change effector function efficiency




⊗ CDC residue ☒ ADCC

FIG.67

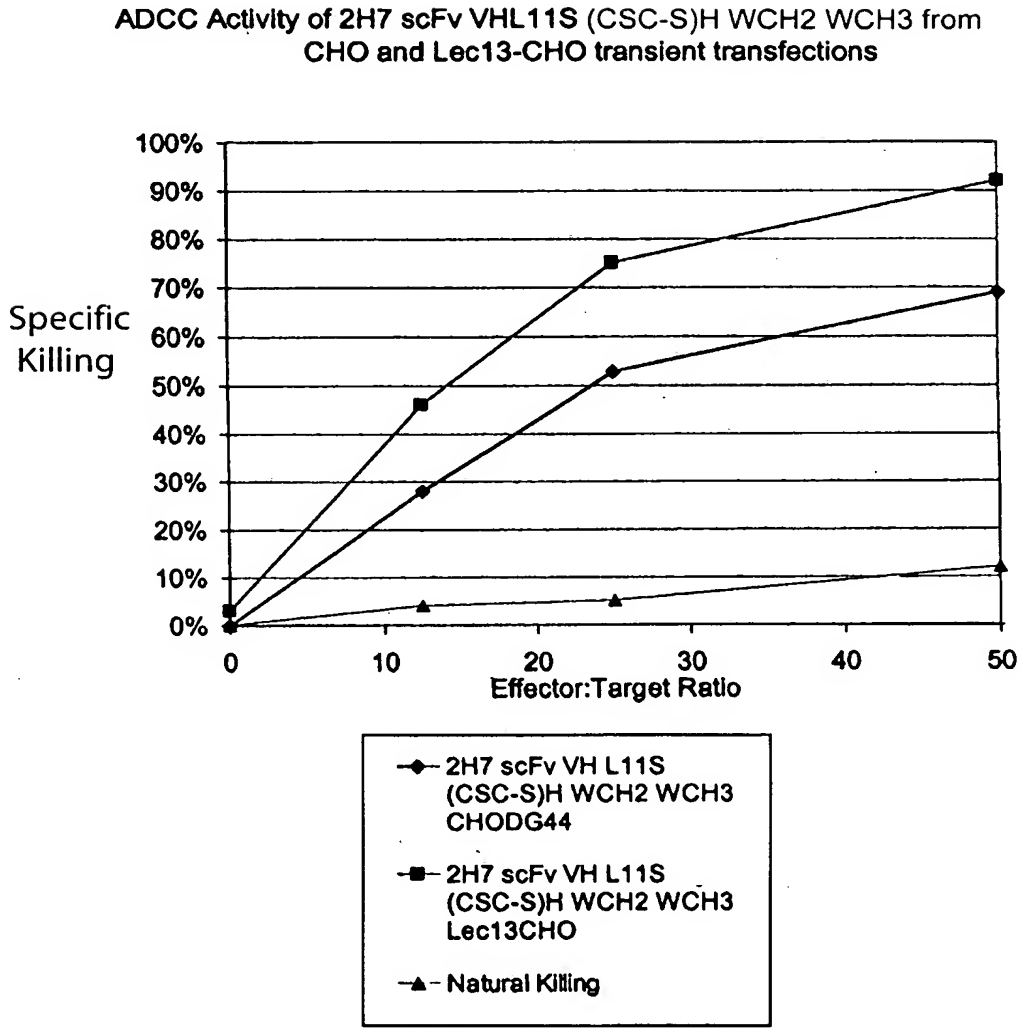

ADCC Activity of 2H7 scFv VHL11S (CSC-S)H WCH2 WCH3 from CHO and Lec13-CHO transient transfections
Specific Killing
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
10
20
30
40
50
Effector:Target Ratio
2H7 scFv VH L11S (CSC-S)H WCH2 WCH3 CHODG44
2H7 scFv VH L11S (CSC-S)H WCH2 WCH3 Lec13CHO
Natural Killing

FIG.68

CD16(ED)(SSS-S)H P238S CH2 WCH3 high and low affinity alleles expressed as soluble molecules

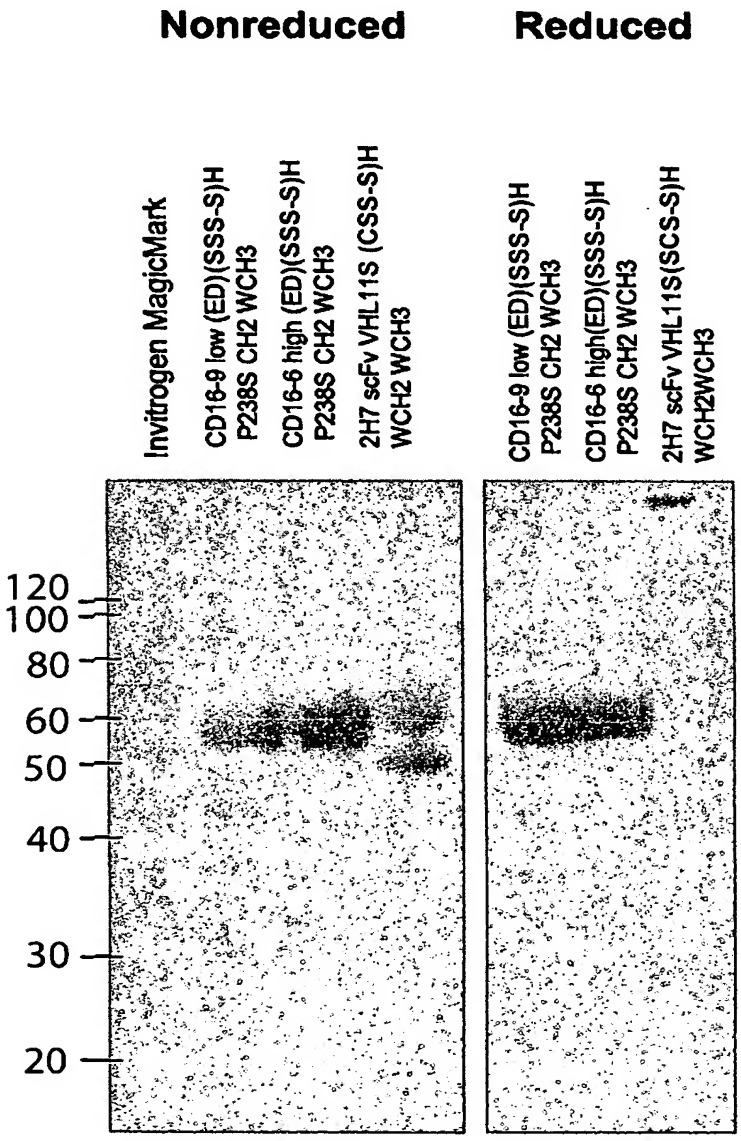


FIG.69

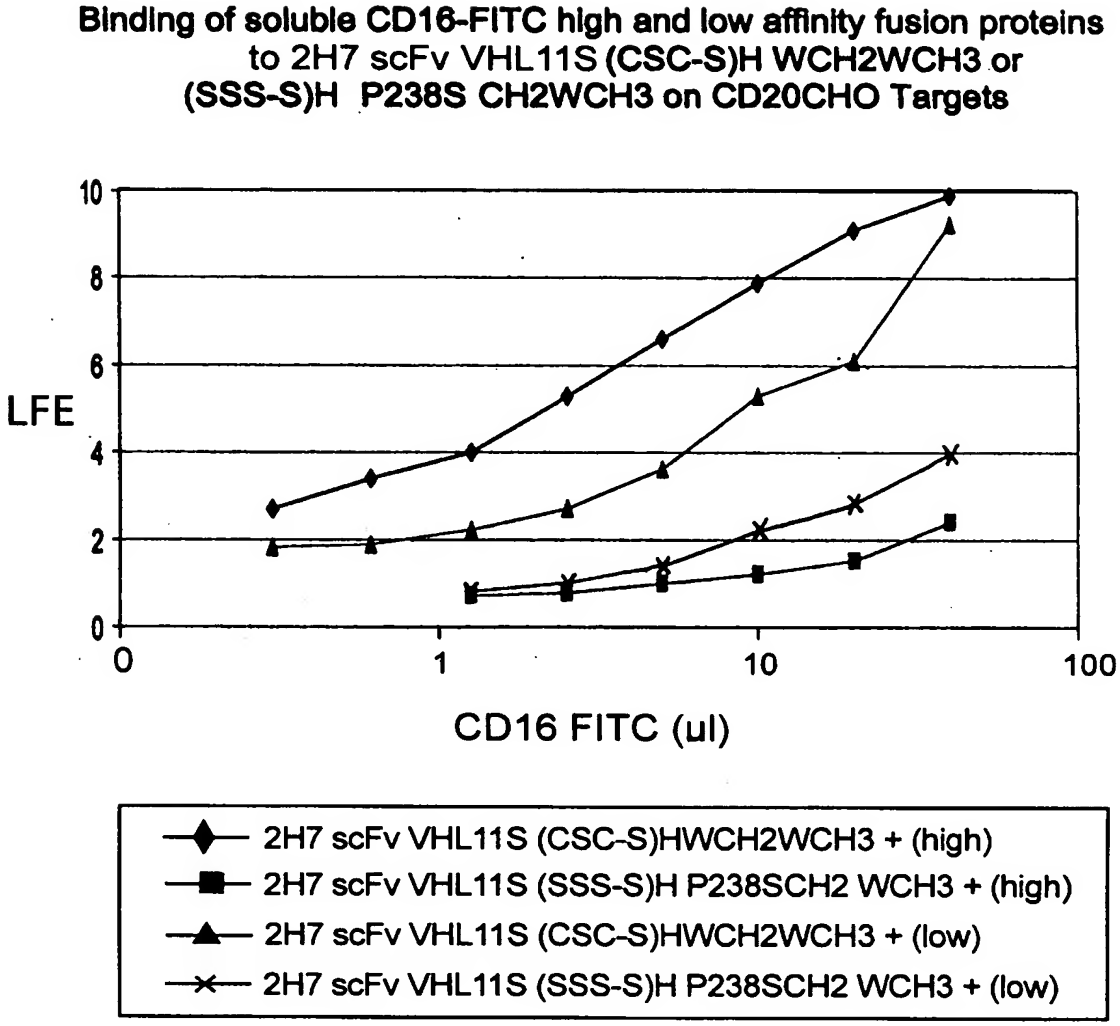
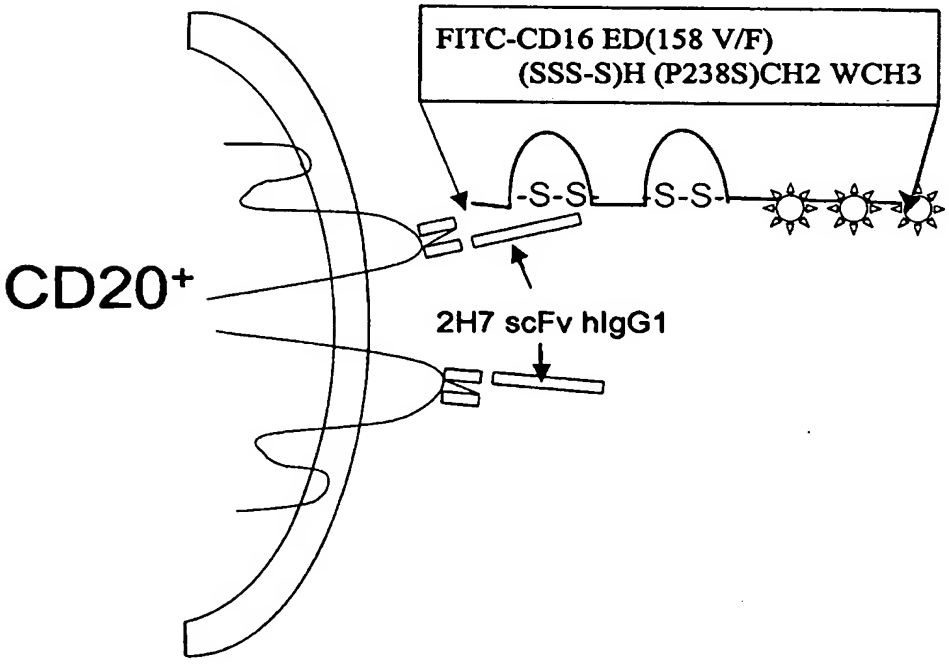
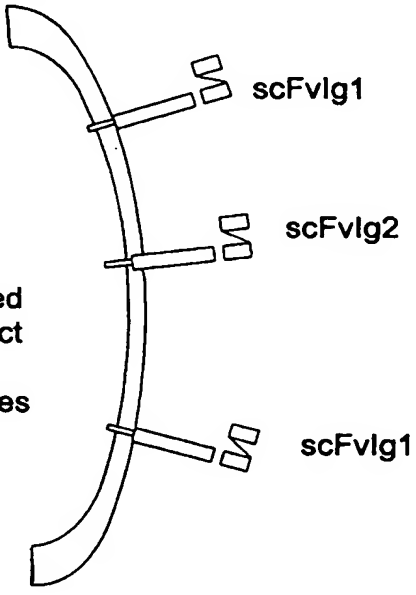

Binding of soluble CD16-FITC high and low affinity fusion proteins to 2H7 scFv VHL11S (CSC-S)H WCH2WCH3 or (SSS-S)H P238S CH2WCH3 on CD20CHO Targets
LFE
10
8
6
4
2
0
0
1
10
100
CD16 FITC (ul)
2H7 scFv VHL11S (CSC-S)HWCH2WCH3 + (high)
2H7 scFv VHL11S (SSS-S)H P238SCH2 WCH3 + (high)
2H7 scFv VHL11S (CSC-S)HWCH2WCH3 + (low)
2H7 scFv VHL11S (SSS-S)H P238SCH2 WCH3 + (low)

FIG.70

Binding of FITC Labeled, Recombinant Human CD16(ED) extracellular domain -Ig Fusion Protein to CytoxB Derivatives on CD20 CHO Cells




Expression of surface displayed SMIPs links modified cDNAs with the altered fusion proteins




Mammalian
Cell Transfected
With
1.A single surface displayed scFvIg expression construct
OR
2.a library of such molecules

FIG.71

# CD37 mAbs and scFvIg Induce Apoptosis

| | Bjab Staining | Annexin V Positive | |
|---|---|---|---|
| | No scFvIg | 17.5 | |
| | 2H7 MH | 27 | |
| scFvIg | G28-1 MH | 30.6 | |
| | G28-1 IgAH | 28.9 | |
| | HD37 MH | 29.1 | |
| | (2H7+G28-1)MH | 41 | |
| | (2H7+HD37) MH | 37.1 | |
| | (G28-1+HD37) MH | 35.3 | |
| | | | |
| | | | |
| | | | plus GAM |
| | Ramos | AnnexinV Positive | AnnexinV positive |
| | cells alone | 3 | 3.3 |
| | 2H7 Mab | 1.4 | 3.1 |
| | G28-1 Mab | 18.3 | 8.7 |
| mAbs | HD37 Mab | 3.7 | 3.1 |
| | G28-5 | 3.9 | 8.3 |
| | 2H7+G28-1 | 32.3 | 35.7 |
| | 2H7+HD37 | 5 | 10.5 |
| | 2H7+G28-5 | 5.7 | 19.4 |
| | HD37+G28-1 | 26.9 | 50 |
| | HD37+G28-5 | 8.2 | 18.4 |
| | G28-1+G28-5 | 39.5 | 68.3 |
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